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Some Properties of Ribonucreic Acid from Yeast 80S Particle; Effect of Magnesium Ions

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(Received for publication, February 20, 1961)

Recently many reports have been issued on the role of magnesium ions in the stability and integrity of the ribosomes (*1–7*). It has been suggested that ability of the ribosomes for the incorporation of amino acid is lost when the concentration of magnesium ions is decreased so as to cause the degradation of ribosomes (*8*). In the previous paper (*9*), the participation of divalent cations in the sedimentation behaviour of the RNA (ribonucleic acid) prepared from yeast ribosomes (80S particle) was presented. The present paper describes further studies on the role of magnesium ions in some properties of the RNA from yeast 80S particles. A possible molecular organization of the RNA is also discussed.

MATERIALS AND METHODS

Matericls—Procedures for preparing the 80S particles from yeast cells and the RNA thereof were essentially the same as previously described (*9*). The procedures were however modified to some extent, especially for in the use of divalent cations. First, the buffer used for the preparations of the 80S particles was 0.002 *M* K-phosphate buffer, pH 6.8, containing 0.001 *M* $MgCl_2$ (*1*). Second, after phenol extraction of the RNA, followed by its ethanol precipitation (*9*), the RNA was dissolved in the above buffer and precipitated again with ethanol. Finally, it was dialyzed against 0.1 *M* NaCl in 0.01 *M* K-phosphate buffer, pH 6.8, containing 0.01 *M* $MgCl_2$

Sedimentation—Sedimentation analyses were made in a spinco ultracentrifuge Model E using a schlieren optical system. Sedimentation coefficients were expressed as Svedberg unit at 20°C, S.

Viscosity—Viscosity measurements were done with an Ostwald viscosimeter at 10.0°C.

Optical Rotation—Optical rotation was measured at 546 mμ in a Rudolph photoelectric polarimeter Model 200 with murcury arc as the light source.

UV-absorbancy—Absorbancy measurements were made in a Hitachi spectrophotometer Model EPB-U.

Determination of the Amount of Magnesium Ions—The amount of magnesium ions in RNA solution was estimated by the titration with EDTA (ethylenediaminetetraacetic acid) using eriochrome black T as the indicator (*10*). This method gives somewhat unclear endpoint when RNA is present. But maximum error anticipated is to be estimated two per cent from the experiments in which a known amount of $MgCl_2$ was titrated in the presence of RNA. The amount of magnesium ions was usually expressed as the atom equivalents of magnesium in solution per mole of phosphate of the RNA (Mg : P). Phosphorus analyses were made by the method of Allen (*11*).

RESULTS

The RNA in SP-M* was dialyzed against SP. At intervals, a portion of the RNA solution was successively taken out and the concentration of magnesium ions was measured. The concentration of magnesium ions decreased continuously to about 0.002 *M* after 10 to 15 hours dialysis as shown in Fig. 1. At this point, excess EDTA, 0.005 *M* in the final concentration, was added into the dialysis bag, and dialysis continued against SP-E for furthur 10 hours.

Sedimentation analyses were made on the final dialysate so obtained as well as on the undialyzed RNA in SP-M. As shown in Fig. 2, two distinct peaks of RNA were present in both preparations. The determina-

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* Following abbreviations are used for the buffer solution for convenience: SP-M, 0.1 *M* NaCl, 0.01 *M* K-phosphate and 0.01 *M* $MgCl_2$, pH 6.8; SP, 0.1 *M* NaCl and 0.01 *M* K-phosphate, pH 6.8; SP-E, 0.1 *M* NaCl, 0.01 *M* K-phosphate and 0.001 *M* EDTA, pH 6.8.

tions of the sedimentation coefficients were done on the sample of the RNA with different concentrations prepared by the addition of each solvent. Sedimentation coefficients were plotted against the concentration to obtain S°. As shown in Fig. 3, the concentration dependency of the sedimentation coefficients is considerable for the fast sedimenting peak, especially for that of the RNA in SP-E. Sedimentation coefficients at zero concentration were estimated as 32S and 19S for the RNA in SP-M and 28S and 19S for the RNA in SP-E, respectively. The value of 32S for the fast sedimenting peak of the RNA in SP-M was larger than 28S for that of the RNA in SP-E or of the RNA described in the previous paper (*9*). The difference seems to exceed over experimental error. In the RNA in SP-M, fast sedimenting peak predominated over slowly sedimenting peak. On the other hand, in the RNA in SP-E the

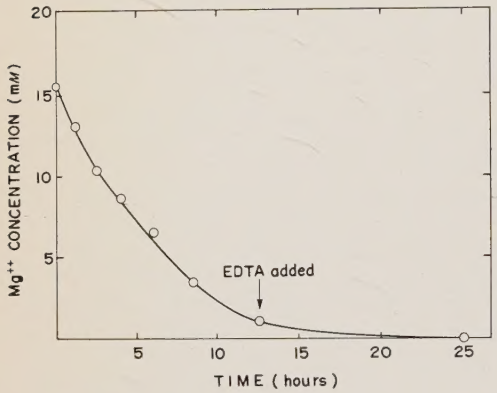


FIG. 1. Change of magnesium ion content in RNA during dialysis against saline.

6.3 mg. per ml. of RNA which had been previously dialyzed against SP-M, was dialyzed against SP with frequent changes of external medium. At the time indicated by arrow, EDTA in final concentration of about 0.005 *M* was added, and dialysis continued against SP-E.

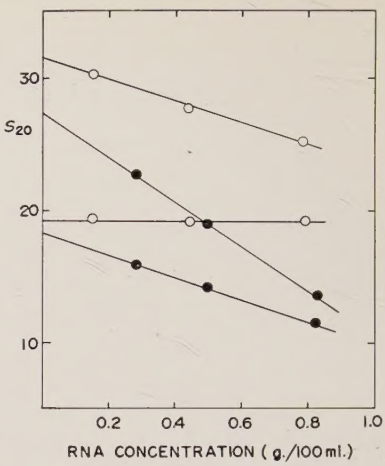
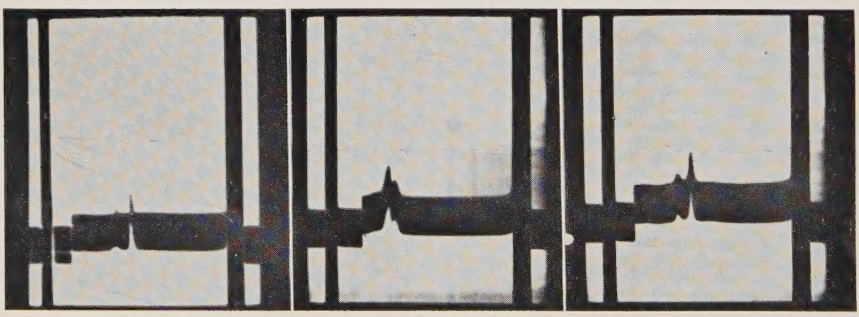


FIG. 3. Dependence of sedimentation coefficients on the concentration of the RNA.
—○— and —●— correspond to (a) and (b) in Fig. 2, respectively.



(a) (b) (c)

FIG. 2. Changes of sedimentation patterns of RNA in 0.1 *M* NaCl in 0.01 *M* K-phosphate buffer, pH 6.8.

(a), RNA was freshly prepared from the ribosomes and dialyzed against solution including 0.01 *M* $MgCl_2$. (b), RNA of (a) was dialyzed in the manner shown in Fig. 1 in the presence of 0.001 *M* EDTA. (c), RNA of (b) was dialyzed against solution containing 0.01 *M* $MgCl_2$. Each pattern is taken on the RNA sample of 8 mg. per ml.

19S peak much increased, and a broad, slowly sedimenting boundary behind 19S peak was observed. Decrease of magnesium ion concentration by dialysis against 0.001 *M* $MgCl_2$ in SP also caused a large increase of the 19S peak. The change of the sedimentation pattern was partially reversible when the magnesium ions were added back (Fig. 2c). Overnight dialysis of the RNA in SP-E against SP-M resulted with the increase in the fast sedimenting peak again. Sedimentation coefficients of the two peaks at infinite dilution were 29S and 20S respectively.

After the RNA in SP-E was dialyzed against distilled water for 48 hours with frequent changes of external water to remove salt, sedimentation patterns were examined on the samples to which various salts were added (Fig. 4). In 0.01 *M* K-phosphate buffer, pH 6.8, a sharp peak with a trace peak that was ahead of the former was observed. With the increase of the concentration of salt, broadening of the peak was seen. In SP, the peak spreaded over the range from 10S to 20S and any sharp boundary could not be identified. By further addition of $MgCl_2$ to it in the final concentration of 0.01 *M*, two broad peaks were observed. On the contrary no appreciable change was observed on the RNA in SP-M in such a treatment. There observed two sharp peaks in which the fast sedimenting one was previaling. Sedimentation coefficients at zero concentration were 18S and 26S. In addition, a small boundary was observed behind the 18S peak.

The magnesium content in the RNA in SP-M decreased continuously when dialyzed against saline (*cf* Fig. 1), whereas it decreased

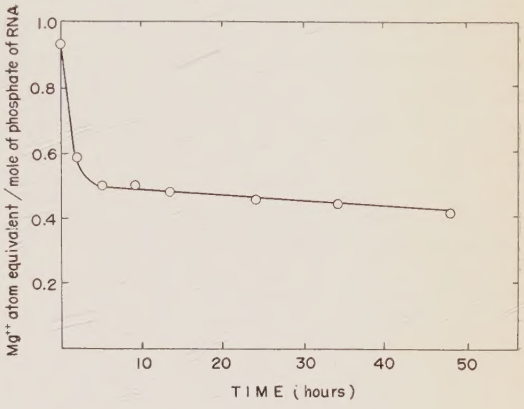
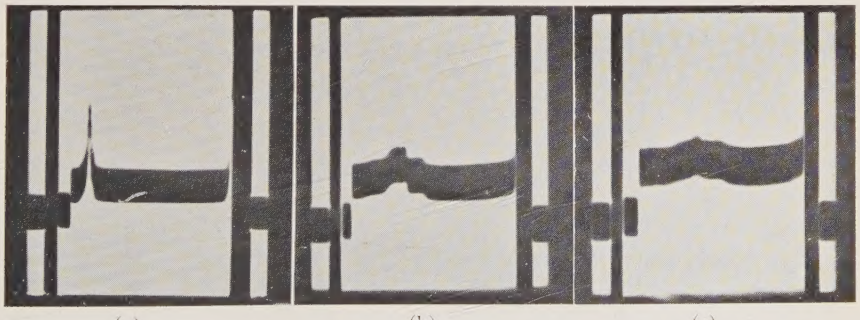


Fig. 5. Changes in magnesium content of RNA during dialysis against distilled water.

6.3 mg. per ml. of RNA dialyzed first against 0.01 *M* $MgCl_2$ in 0.1 *M* NaCl containing 0.01 *M* K-phosphate buffer and then dialyzed against distilled water with the frequent changes of external water.



(a) (b) (c)

Fig. 4. Changes of sedimentation pattern of the RNA with the addition of salt to the RNA previously dialyzed against water.

RNA shown in Fig. 2b was dialyzed against distilled water for 48 hours and then added various solution in the following final concentration, (a), 0.01 *M* K-phosphate buffer, pH 6.8. (b), 0.1 *M* NaCl containing 0.01*M* K-phosphate buffer, pH 6.8. (c), 0.01 *M* $MgCl_2$ in 0.1 *M* NaCl containing 0.01 *M* K-phosphate buffer, pH 6.8. Each pattern is taken on the RNA sample of 4 mg. per ml.

to only a limited extent by the dialysis against distilled water with frequent changes even up to two days. Typical results were shown in Fig. 5. At this steady state Mg : P was consistently about 0.4. A similar Mg : P ratio was also obtained in the experiments described below. First, the RNA of various concentration was dialyzed against 0.01 *M* $MgCl_2$ overnight and then against distilled water as described above*. Second, the RNA (3.3 mg. per ml.) was dialyzed against the solution of $MgCl_2$ with various concentration overnight, and then dialyzed against distilled water. As shown in Table I, the RNA after

TABEL I

The Amount of Residual Magnesium Ions after Dialysis against Distilled Water

RNA concentration (mg. per ml)	Mg : P
6.7	0.43
3.4	0.41
1.8	0.41

RNA of various concentration was dialyzed against 0.01 *M* $MgCl_2$ solution and then dialyzed against distilled water.

$MgCl_2$ concentration in external fluid (m*M*)	Mg : P
10.0	0.41
7.0	0.40
4.0	0.41
1.0	0.39

RNA (3.3 mg. per ml.) was dialyzed against various concentrations of $MgCl_2$ and then dialyzed against distilled water.

the experiment retains almost the same amount of magnesium ions per mole of phosphate of the RNA irrespective of the RNA concentration. In other words, the RNA takes up magnesium ions (Mg : P up to 0.4) which are as a result, not-dialyzable against distilled water.

As has been reported by several authors, the UV-absorption of RNA rised with its denaturation and the phenomenon is generally referred as hyperchromic effect. In the present experiments, the RNA showed the high extent of hyperchromicity depending on Mg : P in the absence of any other added salt. Maximum wave length, however, did not change (Fig. 6). By dialyzing the RNA

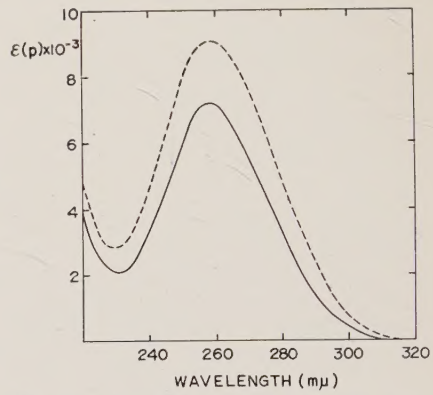


FIG. 6. Absorption spectra in the presence and absence of magnesium ions in distilled water.

RNA of 0.4 (——) and 0.0 (- - - - -) of Mg : P was diluted with water to the final concentration of 20 μg. per ml.. Absorbancy was expressed as the molar extinction for phosphate of RNA.

solution (6.0 mg. per ml.) against 0.1 *M* NaCl, several samples containing various amount of magnesium ions were obtained. They

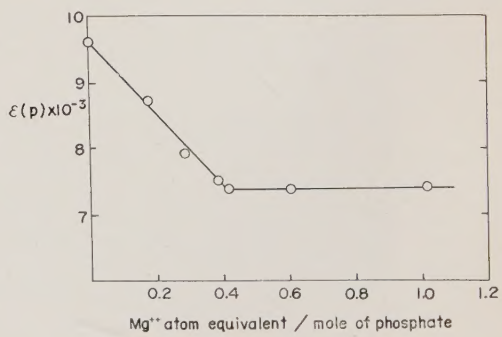


FIG. 7. Dependence of molar extinction coefficient at 260 mμ on the value of Mg : P.

Several samples of various Mg : P was obtained during the dialysis as shown in Fig. 1. These were diltued with water to the final concentration of 20 μg. per ml.

* Usually dialysis was continued until the electric conductivity of the external fluid reached constant value.

were diluted to the final concentration of about 20 μg. per ml. with water and extinction coefficients at 260 mμ per mole of phosphate of RNA, $\varepsilon(p)_{260}$, were determined. As shown in Fig. 7, $\varepsilon(p)_{260}$ was inversely proportional to the value of Mg : P when it is below 0.4, and then constant independently when Mg : P is over 0.4. Moreover, $\varepsilon(p)_{260}$ was almost constant even by furthur addition of NaCl (Table II). Similar results were obtained by the addition of $MgCl_2$ to the RNA containing neither magnesium ions nor any other salt. These facts show that hyperchromicity in the present case was reversible with respect to magnesium ions. 30 per cent increase of $\varepsilon(p)_{260}$ with the removal of magnesium ions was comparable with the heat hyperchromicity described in the previous paper (*9*).

TABLE II

The Changes of Molar Extinction at 260 mμ and Optical Rotation at 546 mμ in the Various Ionic Environments

	ε (p)$_{260}$		$[\alpha]_{546}$	
	0.40	0.0	0.40	0.0
In water	7,400	9,600	210	100
In SP	7,400	7,400	200	190
Alkaline hydrolysate	11,300	11,300	20	20

RNA in SP-M or that freed of Mg^{++} was dialyzed against distilled water.

Alkaline hydrolysis was done in 0.5 *N* KOH for 20 hours at 37°C followed by the neutralization with HCl at the time of measurements.

In Fig. 8, viscosity behaviour of the RNA solution under various conditions was shown. The viscocity of the RNA in SP-M decreased gradually during the succesive determinations in capilary viscosimeter. There did not exist appreciable difference between the viscosity of the RNA in SP-M and SP-E. Viscosity

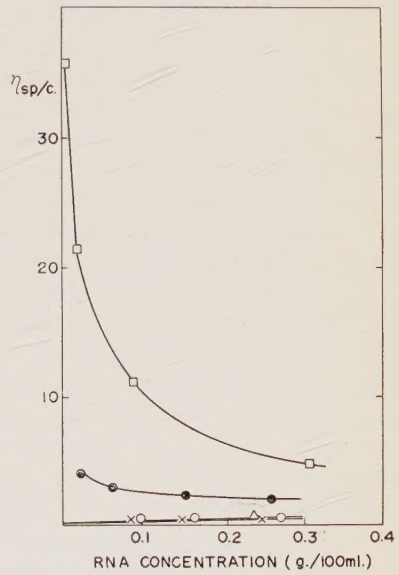
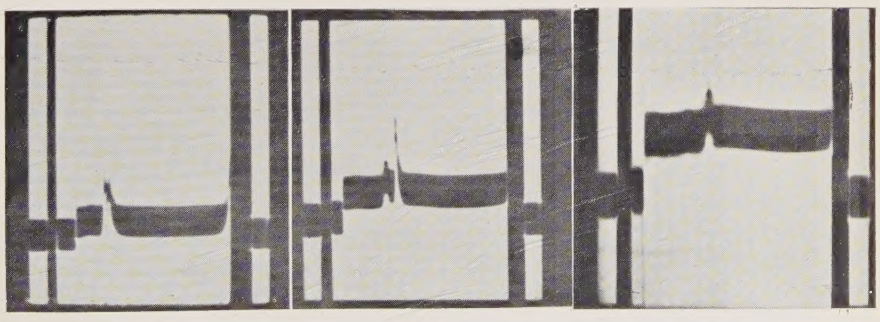


FIG. 8. Dependence of viscosity on the concentration of the RNA in the various ionic environments.

—○— in SP-M, —●— in SP-E (completely freed from magnesium ions), —×— in water, Mg : P is 0.42, —□— in water, Mg : P is 0.0. —△— in $MgCl_2$ solution, Mg : P is 0.72.



(a) (b) (c)

FIG. 9. Sedimentation pattern of the RNA with different Mg : P.
(a), RNA of Mg : P at 0.42 (b), RNA of Mg : P at 0.72 (c), RNA of Mg : P at 1.05.

increased to much higher level after dialysis against distilled water, especially for the RNA in SP-E. Concerning the RNA in SP-M, some rise of viscosity, though not so remarkable as comparing with the case of the RNA in SP-E, was observed by the dialysis against distilled water. The addition of small amount of $MgCl_2$ (Mg : P=0.72) lowered the level of viscosity which is comparable to that of the RNA in SP-M. The similar relationship was observed in sedimentation behaviour. As shown in Fig. 9, the 19S peak predominated at Mg : P of 0.42, whereas it decreased to a trace amount at Mg : P of 0.72. The phenomenon is also observable when a sufficient amount of NaCl was added. At Mg : P of 1.05 aggregation product which ran ahead of any other two peaks was observed.

Table II shows the specific rotation at 546 mμ ($[\alpha]_{546}$) and $\varepsilon(p)_{260}$ of the RNA in the various states. The RNA solution deprived of magnesium ions and well dialyzed against distilled water had a low value of $[\alpha]_{546}$ comparing with that in the presence of sufficient salt or magnesium ions. In the latter case, the value of $[\alpha]_{546}$, which is much higher than the former, was almost constant in the conditions studied.

DISCUSSION

Observations have been made on the reversible dissociation of ribosomal particles from various sources including yeast as the function of the magnesium ion concentration (*1–5*, *7*). In the present experiments a similar reversible process was observed also on the yeast ribosomal RNA. Almost all the sedimentation patterns of the RNA observed under various conditions show two distinct peaks varying greatly depending on the ionic environment. The sedimentation coefficients at zero concentration of each peak differed considerably depending on the conditions. The difference might be caused by the following two reasons. One is the partial degradation of the RNA by the removal of magnesium ions or other salt. The other is the change of hydrodynamic form of the RNA molecules resulted by the ionic environment which whould reflect the sedimentation velocity. With respect to the latter possibility, Boedtker reported (*12*) that sedimentation coefficient of tabacco mosaic virus RNA changes with the conservation of its molecular weight. Considering from her results, it is likely that in our case each corresponding peak of RNA in SP-M and SP-E may be regarded to have the same molecular weight. The fact that the RNA having no magnesium ions in very dilute salt solution sediments very slowly is explained by the charge effect (*5*, *16*). The exact determination of molecular weight can not be made at present. It was, however, shown by several authors (*13–15*) that the relationship between the molecular weight (Mw) and sedimentation coefficient at zero concentration (S°) of RNA can be represented by the empirical formula; $S^\circ = k \cdot M_W{}^\alpha$. Using the value of 0.5_4 as α and $1.3_6 \times 10^{-2}$ as k (*15*) the molecular weight of the 28S RNA and of the 19S RNA was calculated to be $1.4_1 \times 10^6$ and $0.7_1 \times 10^6$, respectively. Thus, the molecular weight of the 28S RNA is as twice as that of the 19S RNA. The results strongly suggest that the 28S RNA is a dimer of two molecules of the 19S RNA. Similar relationship was found by Kurland on *E. coli* ribosomal RNA (*14*).

As mentioned above, magnesium ions play an important role in the integrity of the RNA molecule. The yeast ribosomal RNA possesses the constant amount of magnesium ions (Mg : P=0.4) which is not dialyzable against distilled water. In the presence of a large amount of sodium ions and/or potassium ions, the magnesium ions become dialyzable. Under the conditions where the Mg : P is above 0.4 or in the presence of a large amount of sodium ions, $\varepsilon(p)_{260}$ of the RNA solution has a considerably low value. On the other hand, a marked hyperchromicity is observed when the Mg : P is lowered below 0.4. These changes may well be attributed to the extent of the hydrogen bonds in the RNA molecule (*17–19*). The reverse relationship between the amount of not-dialyzable magnesium ions and the extent of hyperchromicity at the

maximum wave length suggests that the RNA has the specific binding site of magnesium ions to form the hydrogen bond between bases even in the very diluted solution. The binding of magnesium ions to phosphate group was observed in the case of polyphosphate (*20*). The measurements of binding capacity of the RNA to magnesium ions were undertaken by various experimental designs on many kinds of RNA (*5*, *6*, *12*, *21*, *22*) as well as on the synthetic polynucleotide (*23*, *24*). The Mg : P they obtained are comparable with that presented in our experiments. Wacker and Vallee reported (*25*) a considerably lower Mg : P for their RNA preparations. It is likely probable the large proportion of metal was escaped during their preparation process. In the living cells, it is not unlikely that a considerable amount of magnesium ions is bound to RNA molecule.

A considerable dissociation of the dimer RNA occurs to produce monomers below the critical Mg : P (about 0.4), and by further removal of environmental salts the dissociation may be completed. Even at the 0.42 of Mg : P an appreciable amount of the slower component was observed in the absence of excess salt. Generally, RNA in solution behaves as a ramdomly coiled polyelectrolyte. In water, it extends due to the electrostatic repulsion, accompaning with the large increase of viscocity (*13*, *26*). The RNA from the yeast ribosomes also shows the very high viscosity in the absence of salt, especially for the RNA having no magnesium ions. The RNA with 0.42 of Mg : P shows some increase of viscosity when transferred from the salt solution to water, althogh the extent is smaller compared with that of the RNA with that of the RNA with no magnesium ions, was observed. The addition of salt is still required to lower the viscosity to the minimum level.

Optical rotation has been supposed to reflect the helical content in the molecule of RNA (*18*). Dialysis of the RNA in SP-E against distilled water caused the decrease of dextro-rotation. This seems to indicate by the local interruption of hydrogen bonds seem to be reformed by the addition of salt, probably in random fashion. Thus the RNA particles so reformed have various sedimentation rates supposedly with different hydrodynamic forms, giving broad sedimentation boundary of the RNA treated with EDTA in the previous paper (*9*) might be a reflection of this sort of effect.

As discussed, above the isolated RNA from the yeast ribosomes changes its size depending on the concentration of environmental salts. Fundamental unit of the RNA seems to be the 19S RNA (Mw is 0.7×10^6). The presence of salt causes the association of this unit to form dimer, 28S RNA (Mw is 1.4×10^6). Magnesium ions have much remarkable effects on the formation of dimer form as compared with the monovalent ions. The dimer may be constructed by mutual tangling of two subunits of the 19S RNA chains. This becomes possible by the suppression of mutual electrostatic repulsion owing to the neutralization of charges by the salt.

Chao and Schachman observed (*27*) that the RNA occupies about half of the 80S particle (Mw is 4.1×10^6). Chao also observed (*1*) that the 80S particle reversibly dissociated by the removal of magnesium ions into the 60S and 40S particle. Each has molecular weight of two thirds and one third of the 80S particle, respectively. From this point of view, it is supposed that the 60S particle contains two 19S RNA with mutual tangling of each strand and the 40S particle contains one.

Mutual conversion between 19S and 28S RNA was discussed as the dimer-monomer relation. But still following possibilities can not be excluded at the present time. One is that the change of relative ratio of the two components is entirely the reflection of Johnston-Ogston effect (*28*). But this possibility is reduced, because the comparison was made on the patterns taken with the RNA having the same concentration. The other is the reversible denaturation of the RNA as has been indicated by Oth (*29*) in the case of DNA. This is less probable be-

cause there exists the change in sedimentation pattern without any remarkable changes in other properties characteristic to the denaturation of RNA. Further investigations are however required to check these points. At any rate, magnesium ions can bind to the RNA to a considerable extent and in this sense it would take part in maintenance of the structure of the ribosomes.

SUMMARY

The ribosomal RNA prepared from yeast shows two distinct peaks in the sedimentation pattern. The relative ratio of the amount of those two components changes as the function of the amount of salt, especially of magnesium ions. In the presence of 0.1 *M* of NaCl, some portion of the fast sedimenting component converts to slower one as the consequence of removal of magnesium ions. The former is almost completely converted to the latter by further removal of salt by dialysis against distilled water. In this state, the RNA shows very high viscosity, low dextro-rotation and high degree of hyperchromicity.

Considerable amount of magnesium ions binds to the RNA so that they are not dialyzable against distilled water. The amount of magnesium atom equivalents in such form is about 0.4 per mole of phosphate of the RNA. Below this critical amount, a marked hyperchromicity is ovserved in water. Even in the presence of critical amount of magnesium ions, some increase of the slower peak and increase of viscosity are observed without addition of any other salt.

From the above mentioned results, organization of the RNA in yeast ribosomal particle is discussed.

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α-Ketoglutarate Reductase from *Achromobacter*

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The occurrence of various α-hydroxyacid dehydrogenases linking with pyridine nucleotide in many organisms is well known. However, the enzyme responsible for the conversion between α-hydroxyglutarate and α-ketoglutarate has never been reported. During the investigation of the metablolism of L-alloisocitrate in bacteria (*1*), the author found the presence of the enzyme, which was named α-hydroxyglutarate dehydrogenase. But the equilibrium of the reaction is restricted to the direction of reduction of α-ketoglutarate, so the enzyme may be called more properly α-ketoglutarate reductase. The present paper describes some of its properties.

EXPERIMENTALS

Enzyme Preparation from the Bacterium—The bacterium used was *Achromobacter sp.* reported in a previous paper (*1*). The bacterium was grown aerobically with shaking in broth medium for about 20 hours at 30°C. After the harvest from 3 liters of cultures, the bacterium was suspended in 100 ml. of phosphate buffer (0.1 *M*, pH 7.4) and disrupted by sonic oscillater (10 KC) for 5 minutes. From the crude extract, protein was precipitated twice with ammonium sulfate between 30 to 45% saturation. After the precipitated protein was dissolved in 20 ml. of distilled water, calcium phosphate gel was added to the solution (final volume of the solution; 36 ml., concentration of calcium phospahate gel; 8.6 mg./ml.), and the pH was adjusted to pH 6.0. The supernatant was dialyzed against distilled water for 16 hours. Recovery of the enzyme activity was about 33 per cent. The specific activity against optical density at 280 mμ rised for 48.6 fold. This preparation was used throughout this work.

Materials—DPN and TPN were purchased from Mann Research Laboratories Co. Ba-salt of reduced DPN was enzymically prepared with alcohol dehydrogenase (*2*). Reduced TPN was also prepared enzymically with isocitrate dehydrogenase (*2*). L-α-Hydroxyglutaric acid was synthesized by the method of Fischer *et al.* (*3*). According to the polarimetry, the purity was calculated to be almost 100 per cent.

Assays and Determinations—The activity of the enzyme was measured by following the oxidation of DPNH at 340 mμ in a cuvette containing the components of α-hydroxyglutarate dehydrogenase assay system (*1*). The unit of the enzyme activity is defined as described in the previous paper.

Estimation of α-ketoglutarate was done according to the method of Friedeman and Haugen (*4*). Estimation of α-hydroxyglutarate was carried out as follows; The reaction mixture was treated with excess amount of 2,4-dinitrophenylhydrazine, and the hydrazone of the residual α-ketoglutaric acid and excess hydrazine were removed by active charcoal. After the removal of α-ketoglutarate, α-hydroxyglutaric acid was extracted from the solution with ether by continuous extraction for 48 hours. The extracted acid was oxidized with acidic $KMnO_4$ in a Warburg vessel and the amount of CO_2 evolved was measured manometrically (*5*).

RESULTS

Reduction of α-Ketoglutarate—As shown in Fig. 1, the decrease of optical density at 340 mμ was observed after the addition of α-ketoglutarate. Plot of enzyme concentration against initial decrease in optical density at 340 mμ was linear as far as studied. Reversibility of the reaction has been proved in the previous paper.

Identification of Product—The formation of α-hydroxyglutaric acid from α-ketoglutaric acid was proved paperchromatographically as reported in the previous paper. The optical rotatory power of the molybdate complex of the acid was measured according to the method of Krebs *et al.* (*6*). The $[\alpha]_D$ value was observed to be −95° (the value

for synthetic L-α-hydroxyglutaric acid; −107.5°).

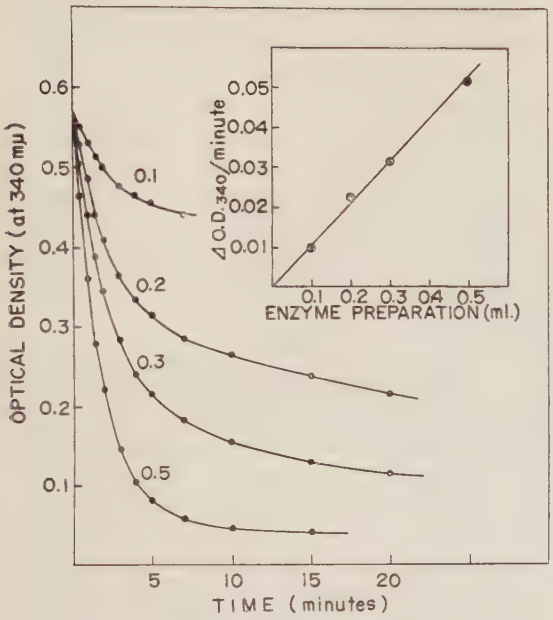

Fig. 1. Optical assay of α-ketoglutarate reductase. 3.0 ml. of reaction mixture containing; phosphate buffer, pH 7.4, 260 μmoles; DPNH, 0.3 μmoles; sodium α-ketoglutarate, 1.0 μmoles; the enzyme preparation as indicated in the figure.

Stoichiometry—The data in Table I indicate a 1:1 ratio between the disappearance of α-ketoglutarate and DPNH and the formation of α-hydroxyglutarate.

α-Ketoglutarate + DPNH + H^+ → α-hydroxyglutarate + DPN^+

Table I

Stoichiometry of α-Ketoglutarate Reduction

In a final volume of 15 ml., the following components were incubated at 30°C for 60 minutes; Tris buffer, pH 7.4, 250 μmoles; DPNH, 67 μmoles; α-ketoglutarate, 100 μmoles; the enzyme preparation, 1.5 ml.

α-Ketoglutarate disappeared	DPNH disappeared	α-hydroxyglutarate formed
66.1 μmoles	66.7 μmoles	61 μmoles

Effect of pH—The maximal activity for the reaction was found near pH 7.0 in Tris buffer, and between pH 6–8 in phosphate buffer. The pH-activity curve varied markedly depending on the kinds of buffers as shown in Fig. 2.

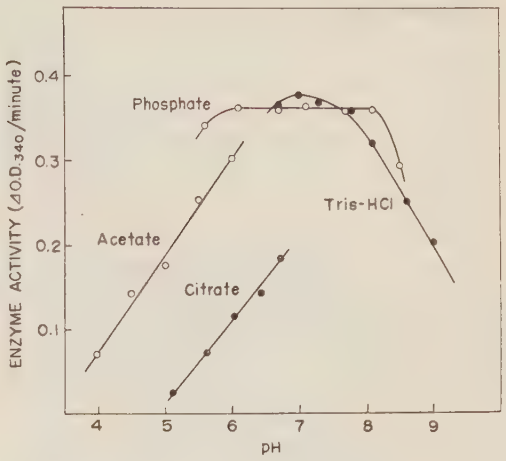

Fig. 2. Effect of pH on the reduction of α-ketoglutarate.

Effect of Substrate Concentration—Initial rates of α-ketoglutarate reduction at several concentration of α-ketoglutarate and DPNH were measured in phosphate buffer at pH 7.4. From the reciprocal plot shown in Fig. 3, the Michaelis-Menten constants were calculated to be $5\times10^{-5}M$ for α-ketoglutarate and $1.3\times10^{-4}M$ for DPNH, respectively.

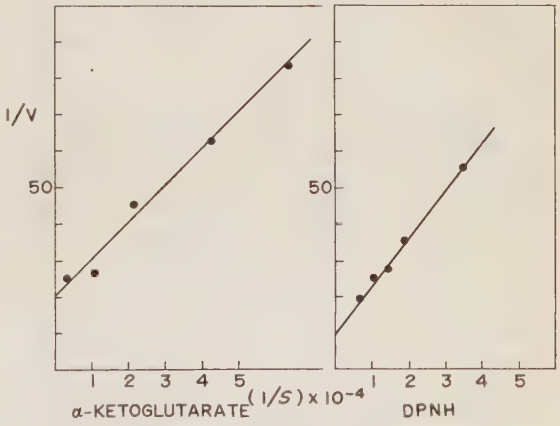

Fig. 3. Effect of substrate concentration on the velocity of α-ketoglutarate reduction.

Effect of Inhibitors—*p*-Chloromercuribenzoate, $10^{-4}M$, inhibits the enzyme activity for

63 per cent. The following chemicals are without effect at $10^{-3}M$; EDTA, 8-hydroxyquinoline, thiourea, NH_2OH, monoiodoacetate and NaF.

Effect of Metal—After the dialysis against EDTA ($3\times10^{-3}M$) for 16 hours, the enzyme activity decreased by about 35 per cent, which was not reversed by the addition of Mn^{++} nor Mg^{++} at $10^{-4}M$ concentration. Though the activation of this enzyme by the metals was reported in the previous paper, it must be corrected.

Substrate Specificity—The final enzyme preparation still contained the activity of DPN-linked malate dehydrogenase, but its ratio to α-ketoglutarate reductase activity varied markedly during the purification (Table II).

Table II

Substrate Specificity of the Enzyme

Substrates		Specific enzyme activity (unit/ml./O.D.$_{280}$)	
α-ketoacid	Pyridine nucleotide	Crude extract	Purified prep.
α-Ketoglutarate	DPNH	0.315	15.3
//	TPNH	—	1.5
// $+NH_3$	//	0.173	8.5
Oxalacetate	DPNH	10.9	11.5
Pyruvate	//	0.023	0.47
Glyoxylate	//	0.023	0.24

It was also proved by the "mixed-substrate" method that α-ketoglutarate reductase is functionally quite distinct from TPN-linked glutamate dehydrogenase. When the substrates for both α-ketoglutarate reductase and glutamate dehydrogenase (i.e., TPNH, DPNH, α-ketoglutarate and ammonium ion) were added together at their enzyme-saturating concentrations, the total oxidation rate of reduced pyridine nucleotides was the sum of the rates of the reactions measured separately. From these data, the enzyme seemed to be specific for α-ketoglutarate and DPNH. Weak activity observed with TPN may be elucidated, at least partially, by the presence of TPN-linked glutamate dehydrogenase and slight amount of ammonium ion in the preparation.

DISCUSSION

Muscle lactate dehydrogenase has been found to be active on a large number of keto acids, except for α-ketoglutarate (*7*). And the enzyme catalyzing direct reduction of α-ketoglutarate, one of the most common keto acids in the cell, has never been reported. The results described in this paper prove the occurreence of a specific α-ketoglutarate reductase. It is possible that the enzyme plays a role in regeneration of oxidized DPN *in vivo*.

SUMMARY

A diphosphopyridine nucleotide-linked α-ketoglutarate reductase has been found from *Achromobacter sp.* The enzyme catalyzes reversible conversion between α-ketoglutarate and L-α-hydroxyglutarate, but the equilibrium of the reaction is very restricted to the direction of formation of the latter. The reaction proceeds at maximal velocity between pH 6–8 in phosphate buffer. Half maximal rate was obtained at a DPNH concentration of $1.3\times10^{-4}M$ and a α-ketoglutarate concentration of $5\times10^{-5}M$. The enzyme seemed to be specific for DPN and L-α-hydroxyglutarate. The enzyme was inhibited by *p*-chloromercuribenzoate.

The author wishes to express his gratitude to Prof. K. Arima, for his kind advice during the course of this work.

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Studies on Laccases of Lacquer Trees

III. Reconstruction of Laccase from Its Protein and Copper

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(Received for publication, May 2, 1961)

In order to conclusively establish the essential role played by copper in the catalytic activity of laccase, it is desirable to demonstrate that the removal of the copper completely inactivates the enzyme and that the copper-free enzyme can be specifically reactivated by the addition of copper ion. Experiments along this line were in fact carried out in 1948 by Tissières (*1*) who obtained inactive, copper-free laccase by treating the enzyme with cyanide in the presence of catechol and ascorbic acid, and showed that the addition of cupric ion, but not of iron, manganese and cobalt ions, restored the lost activity.

In a previous paper of this series (*2*) it was reported that the copper is very firmly bound to the laccase molecule when it is in the oxidized form and no exchange takes place between the enzyme-bound copper and exogenous cupric ion. It was, however, found that the copper-protein linkage becomes considerably labile in reduced laccase so that the copper can freely exchange with added cuprous ion, although it can not be readily replaced by cupric ion. In view of these findings, it seemed of considerable importance to reinvestigate Tissières' reconstruction experiments in order to obtain further insight into the state and function of copper in the laccase molecule.

This paper describes experiments in which attempts were made to remove the copper from laccase and to reconstruct the active enzyme from the copper-free protein and copper ions.

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EXPERIMENTAL

Preparation of Rhus vernicifera Laccase—Rhus vernicifera laccase was purified as described previously (*3*). All the enzyme preparations used in this study were electrophoretically and ultracentrifugically pure, though their specific activity and copper contents varied somewhat from preparation to preparation.

Dialysis of Laccase—All the dialysis experiments were carried out in a cold room maintained at 4°C using Visking cellophane tubings. The stock solution of purified *R. vernicifera* laccase in distilled water was diluted with either acidic buffer or chelating agent solution to make the protein concentration to about 1 per cent, and then dialyzed against the acidic buffer or the chelating agent solution for about 20 hours with gentle stirring of the external fluid. After the dialysis, the buffer salts or the chelating agent were removed from the laccase solution by prolonged dialysis against distilled water. The buffers and chelating agents used were as follows; 0.2 *M* sodium acetate-acetic acid buffer of pH 6.0—4.0, 0.1 *M* sodium citrate-hydrochloric acid buffer of pH 4.0—1.0, ethylenediamine tetraacetate (EDTA) solution in 0.1 *M* phosphate buffer of pH 7.0, and potassium cyanide solution in 0.1 *M* phosphate buffer of pH 8.0

Cuprous Chloride Solution—Cuprous chloride solutions used in the reconstruction experiments were prepared by reducing cupric sulfate with ascorbic acid under anaerobic conditions. A dilute solution of cupric sulfate in 0.5 *M* acetate buffer, pH 5.5, containing 2 per cent of sodium chloride was placed in the main room of a Thunberg tube. The side arm received an aqueous solution of L-ascorbic acid five times in excess for completely reducing the added cupric salt. After the tube was repeatedly evacuated and flushed with pure nitrogen, the contents of both main room and side arm were mixed. Cupric sulfate was immediately reduced to form colorless cuprous chloride and remained stable under anaerobic conditions.

Moderate concentrations of sodium chloride were added to the system not only to stabilize the cuprous chloride solution but also to increase the solubility of cuprous chloride in the buffer.

Other Procedures—The determination of copper and the measurement of laccase activity were performed as described in the preceding paper (*3*). When the activity of laccase had to be measured in the presence of excess cupric ion, 0.2 *M* acetate buffer of pH 6.0 was used instead of 0.13 *M* phosphate buffer of pH 7.0. The copper-content and specific activity of the enzyme preparations were expressed on the dry weight basis.

Chemicals—All the chemicals used in the following experiments were of analytical grade, and the water used was twice distilled in an all-glass apparatus.

RESULTS

Dialysis against Acidic Buffer—Table I shows that the dialysis of laccase against 0.2 *M* acetate buffer of pH 6.0, 5.0, and 4.0 for about 20 hours caused no loss in the copper content and the catalytic activity. The absorption spectrum of the enzyme was also unaffected. These results are in accord with the copper exchange studies reported in a previous paper (*2*) in which it was reported that the copper of laccase was firmly bound to the protein in the oxidized state. Though the diffusion of cupric ion through cellophane membrane was relatively slow, it was confirmed in preliminary experiments that dialysis against the acidic buffer for 20 hours followed by dialysis against distilled water for 40 to 60 hours was sufficient to completely remove free cupric ion from the enzyme solution.

TABLE I

Dialysis of Laccase Solutions against 0.2 M Acetate Buffers of Varying pH Values

Exp. No.	pH of buffer	Dialyzed laccase: Specific activity Q_{O_2}	Dialyzed laccase: Copper content %
Standard.	—	7.55×10^3	0.250
1	6.0	7.47× 〃	0.249
2	5.0	7.70× 〃	0.253
3	4.0	7.45× 〃	0.245

When laccase solution was dialyzed against 0.1 *M* citrate buffer of higher acidity, the copper was found to dissociate from the enzyme as shown in Table II. The critical pH, above which the copper of the enzyme did not appreciably dissociate from the protein, was around 2.5. The catalytic activity and the blue color of the enzyme were also lost in parallel to the decrease of the copper content. The copper-free laccase obtained by dialysis against 0.1 *M* citrate buffer of pH 1.4–1.6 will henceforth be called acid-dialyzed apo-laccase. The apo-laccase did not precipitate from the solution after prolonged dialysis against distilled water, and was almost colorless.

Dialysis against Chelating Agent Solution—Dialysis against EDTA solutions failed to remove the copper from laccase (Table III). This again lends support to the previously presented conclusion that the affinity between the copper and the protein in oxidized laccase is very high.

Dialysis against potassium cyanide solutions removed copper from the enzyme almost completely concomitant with the loss of the blue color and the catalytic activity (Table III). As the copper must have been removed from the enzyme in the form of very stable cuprous cyanide complexes, cyanide ion acted upon the copper as a reducing agent as well as a complex-forming agent. The copper-free

TABLE II

Dialysis of Laccase Solutions against 0.1 M Citrate Buffers of Varying pH Values

Exp. No.	pH of buffer	Dialyzed laccase: Specific activity Q_{O_2}	Dialyzed laccase: Copper content %
Standard.	—	9.10×10^3	0.250
1	4.0	8.70× 〃	0.250
2	2.8	8.95× 〃	0.240
3	2.3	7.10× 〃	0.236
4	1.9	3.80× 〃	0.146
5	1.7	1.29× 〃	0.053
6	1.4	0.65× 〃	0.005

TABLE III

Dialysis of Laccase Solutions against Copper-Chelating agents Solutions

Exp. No.	Chelating agents solutions	Dialyzed laccase: Specific activity Q_{O_2}	Dialyzed laccase: Copper content %
Standard.	—	7.05×10^3	0.245
1	$1\times10^{-2}M$ EDTA in 0.1 M phosphate buffer of pH 7.0	$6.84\times$ //	0.235
2	$1\times10^{-2}M$ KCN in 0.1 M phosphate buffer of pH 8.0	$0.44\times$ //	0.011
3	$2\times10^{-3}M$ KCN in 0.1 M phosphate buffer of pH 8.0	$0.39\times$ //	0.010

laccase thus obtained will be designated as cyanide-treated apo-laccase in this paper. It was almost colorless, but in some preparations faint yellow tint was observed (Fig. 1).

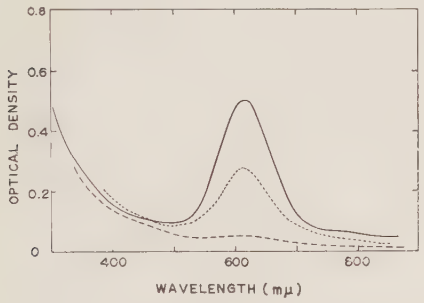

FIG. 1. Absorption spectra of native, cyanide-dialyzed, and reconstructed laccases. 1.00% solution in distilled water. Optical path, 1 cm.
—— Native laccase
– – – Cyanide-dialyzed apo-laccase
..... Partly reconstructed laccase

Reconstruction Experiments—Having succeeded in removing the copper from laccase by acid and cyanide treatments, it was attempted to reconstruct active enzyme from copper ions and the inactive apo-laccases. Only copper ions were employed in these attempts, since it had been clearly shown that copper is specifically required for the laccase activity (*1*).

The results obtained in preliminary experiments indicated that the addition of cupric sulfate to the reaction mixture was completely ineffective in restoring the catalytic activity of both acid- and cyanide-treated apo-laccases. Since this finding was not in agreement with Tissières' report on the restoration of the oxidase activity of apo-laccase by the addition of cupric sulfate (*1*), it was decided to study the problem more carefully.

Thus, the apo-laccase solution in acetate buffer of pH 6.0 was mixed with a solution containing an excess of either cupric sulfate or cuprous chloride and the mixture was kept at 4°C for 2 hours; the incubation with cuprous chloride being carried out anaerobically in a Thunberg tube in order to avoid the oxidation of cuprous copper. After incubation, the mixture was dialyzed against the buffer and then against distilled water to remove free copper ions and buffer salts. The catalytic activity and copper content of the dialyzed solution were then determined. The results of these determinations are recorded in Table IV.

TABLE IV

Reconstruction of Laccase from Apo-laccases and Copper

	Specific activity Q_{O_2}	Copper content %
Native laccase	7.55×10^3	0.250
// + Cu^{++}	$8.40\times$ //	0.249
// + Cu^{+}	$8.10\times$ //	0.265
Acid-treated apo-laccase	$1.11\times$ //	0.038
// + Cu^{++}	$0.87\times$ //	0.588
// + Cu^{+}	$0.87\times$ //	0.554
Cyanide-treated apo-laccase	$0.19\times$ //	0.003
// + Cu^{++}	$0.24\times$ //	0.135
// + Cu^{+}	$2.38\times$ //	0.182

It is clear from these data that the oxidase activity of cyanide-treated apo-laccase could be partly restored by the incubation with cuprous ion and this restoration of activity was accompanied by an increase in the content of protein-bound copper. As can be seen from Fig. 1, it was further confirmed that the blue color characteristic of native

laccase was also partly restored by the incubation. The copper content of cyanide-treated apo-laccase could also be increased when incubated with cupric sulfate. No restoration of the activity was, however, observed in this case and the enzyme solution remained colorless. It may therefore be concluded that the observed incorporation of cupric copper was not due to the restoration of the specific copper-protein linkage in native laccase.

The catalytic activity of acid-treated apo-laccase, on the other hand, could not be increased by the contact with either cuprous or cupric ion. Both cuprous and cupric ions were, however, incorporated into the apo-laccase and the copper contents determined after the incubation were more than twice as high as that of native laccase. It is very likely that the enzyme protein has undergone denaturation during the acid treatment and certain polar groups capable of binding copper have been exposed. Native laccase showed no tendency to combine excess copper, but its specific activity was slightly increased by the contact with both cuprous and cupric ions.

The fact that only partial restoration of the activity was accomplished with cyanide-treated apo-laccase does not seem to be accounted for by the slow incorporation of cuprous ion under the conditions employed, since prolonged incubation with cuprous chloride did not further increase the restored activity. It appears more probable to assume that the enzyme protein has suffered some modifications in the course of cyanide dialysis. This possibility is also supported by the fact that the apo-laccase binds appreciable amounts of copper by the incubation with cupric ion, though the activity cannot be restored by this treatment. The cyanide-treated apo-laccase was, however, found to be ultracentrifugically homogenous (Fig. 2), and its sedimentation coefficient was nearly the same as that of native laccase.

DISCUSSION

The copper in the laccase molecule could be removed by dialyzing the enzyme solution against 0.1 *M* citrate buffers of pH values lower than 2.5. But, in this case, the dissociation of copper from the enzyme seems to be accompanied by the denaturation of the enzyme-protein as evidenced by the failure of the apo-protein thus obtained to regain any catalytic activity by the addition of cupric and cuprous ions.

The copper of laccase could also be removed almost completely by cyanide-dialysis, a procedure which has been most commonly used in preparing apo-proteins from copper-containing enzymes. The activity of cyanide-treated apo-laccase was found to be partly restored by the contact with cuprous ion, but cupric ion was ineffective. This finding was

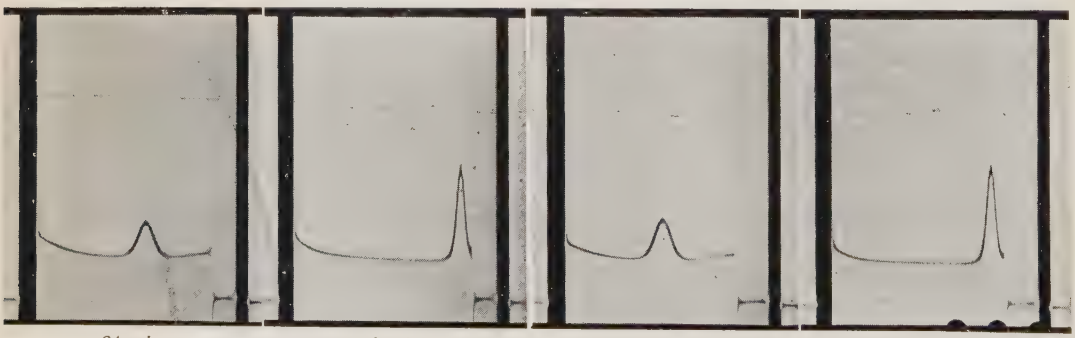

FIG. 2. Sedimentation patterns of native laccase (right) and cyanide-dialyzed apo-laccase (left). Protein concentrations, 0.42 g./100 ml. (right), and 0.49 g./100 ml. (left). Rotor speed, 59,780 r. p. m. Rotor temperature, 11.0°C (right), and 10.0°C (left). Corrected sedimentation coefficient (S_{20w}) calculated is 5.81 S for native laccase, and 5.63 S for cyanide-dialyzed apo-laccase.

rather unexpected in view of the fact that the copper of laccase can exist in either cupric or cuprous state (*4*), and of Tissières' work on the reconstruction of active laccase from apo-laccase and cupric ion (*1*). It should be mentioned in this connection that the cyanide-treated apo-laccase employed in this study was differently prepared from that used by Tissières who removed the copper by the action of cyanide on reduced laccase in the presence of catechol and ascorbic acid.

Two other copper-containing oxidases, potato tyrosinase (*5*) and cucumber ascorbic acid oxidase (*6*), have been successfully reconstructed from their cyanide-treated apo-proteins and copper. Cupric ion was effective in these cases. Hemocyanin, on the other hand, has been shown to require cuprous copper for the recombination of copper-free apo-protein with the metal (*5*, *7*). The copper of hemocyanin, however, seems to exist always in the cuprous state (*8*). It is therefore rather unusual that the reconstruction of laccase requires cuprous ion instead of cupric copper.

A reasonable explanation, however, appears to be provided by the copper exchange studies reported in a previous paper (*2*). It was shown in these studies that cupric ion cannot enter the reduced laccase molecule to replace the labile cuprous copper of the enzyme. Such a behavior of cupric ion is in accord with its inability to restore the catalytic activity of cyanide-treated apo-laccase.

Another possibility may be that the effective reconstruction with cuprous chloride is due to the reducing power of excess ascorbic acid contained in the cuprous chloride solution employed. It is conceivable that the functional groups responsible for the specific copper-protein linkages are exposed by the removal of the copper and reversibly oxidized to inactive forms by atmospheric oxygen. Ascorbic acid is very likely able to reduce these oxidized groups. The technique of anaerobic dialysis may be useful to clarify this point. Such experiments are desirable to obtain information on the functional groups of the laccase protein which binds copper so strongly.

SUMMARY

1. The copper of laccase was removed from the enzyme by dialysis against acid and cyanide, and colorless apo-enzymes were obtained.

2. The catalytic activity of the apo-enzyme prepared by acid dialysis was not restored by the addition of cupric and cuprous ions.

3. The catalytic activity of cyanide-dialyzed apo-laccase was partly restored by the contact with cuprous ion, but cupric ion was ineffective. The characteristic blue color of laccase was also partly restored by the recombination of apo-enzyme and cuprous ion.

The author wishes to express his hearty gratitude to the late Prof. A. Nishimura for his interest and encouragement during this investigation, and to Prof. Y. Ogura, University of Tokyo, for his valuable advices. Thanks are also due to Mr. S. Yamamoto, the Institute for Infectious Diseases, University of Tokyo, for sedimentation measurements, and to Miss A. Kurono for her co-operation in this work. The author also expresses his gratitude to Saito Co. Ltd. for the kind supply of lacquer latex.

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Studies on Laccases of Lacquer Trees

IV. Purification and Properties of a Blue Protein Obtained from Latex of *Rhus vernicifera*

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The presence of a blue pigment in the laccase preparations obtained from lacquer trees has been described by Keilin and Mann (*1*). They separated the pigment from their purified laccase preparations, and reported that the pigment was free of copper and protein but was always mixed with carbohydrates. This pigment was considered to be involved in the normal activity of the enzyme, but its nature has been left unelucidated.

In a previous paper of this series (*2*), it was shown that a blue-colored protein lacking laccase activity is present in the latex of Japanese lacquer tree (*Rhus vernicifera*). As the intense blue color of this new protein suggested its similarity to the pigment reported by Keilin and Mann, it was attempted to further purify this blue protein. This paper reports the purification procedure of the blue protein as well as some of its properties. The blue protein is hereafter called *R. vernicifera* blue protein.

EXPERIMENTAL

Purification Procedure of R. vernicifera Blue Protein— As already reported in a previous report (*2*), *R. vernicifera* blue protein was separated from *R. vernicifera* laccase at the final step of the purification procedure of the enzyme, *i.e.* at the step of ion-exchange chromatography on an Amberlite XE-64 column. When the column was treated with 0.05 *M* sodium phosphate buffer, pH 5.5, the blue-protein formed a strongly blue colored band remaining at the top of the column, and the band was clearly separated from the broad blue band of laccase by a narrow yellow band. The resin bearing the blue protein was cut off from the column and eluted with cold 0.2 *M* Na_2HPO_4. The blue protein was then precipitated from the eluate by the addition of 1.2 volume of cold acetone, dissolved in a few milliliters of distilled water, and dialyzed against distilled water to remove sodium phosphate.

The dialyzed blue solution obtained from 5 kg. of crude latex was mixed with an equal volume of 0.2 *M* sodium phosphate buffer of pH 6.0. The solution was then applied to a 3×40 cm. column of Amberlite XE-64 which had been equilibriated with 0.1 *M* sodium phosphate buffer of pH 6.0, and the column was eluted with the same buffer. The blue band formed at the top of the column moved slowly downwards, and a small amount of yellow impurities moved faster. When the blue band had migrated about 10 cm., the elution was stopped. The blue band was cut off from the column, and eluted with cold 0.2 *M* Na_2HPO_4. The purified blue protein was again precipitated from the eluate with acetone, dissolved in distilled water, and dialyzed against distilled water.

The blue protein could also be precipitated from the solution by the saturation with ammonium sulfate. It was, however, far less readily precipitated from the solution than laccase, and the ammonium sulfate precipitation was always accompanied by a considerable loss of the protein.

The yield of the blue protein was considerably variable, though that of laccase was almost constant. The blue protein content in crude latex therefore seems to be subject to some sorts of variations; the two batches of latex which were rich in the blue protein were proved to have been collected in autumn, and the other two batches collected in early summer contained relatively small smounts of the blue protein. Whether or not such is generally the case should be proved by repeated examinations of lacquer latexes for years. The best yield was about 150 mg. of purified

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blue protein from 5 kg. crude latex. The latex of Chinese lacquer tree (*Rhus vernicifera*) contained only small amounts of the blue protein, and the content of laccase was also lower than that of Japanese lacquer tree.

Other Procedures—The detection and determination of copper were preformed as described previously (*2*). The oxidase activity was determined by measuring the rate of oxygen uptake by the conventional manometric method.

RESULTS

Criteria of Purity—Sedimentation measurements of the purified blue protein were carried out in a Spinco model E analytical ultracentrifuge. The protein showed only a single peak indicating its ultracentrifugical homogeneity (Fig. 1). Electrophoretical characteristics of the protein were also studied by using a Hitachi model HTB Tiselius-type apparatus. As in shown in Fig. 2, a single boundary was obtained indicating the electrophoretical homogeneity of the purified preparation.

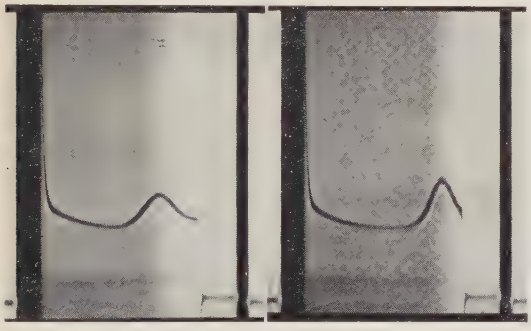

FIG. 1. Sedimentation patterns of *R. vernicifera* blue protein in 0.1 *M* phosphate buffer of pH 6.5. Protein concentration, 0.35 g./100 ml. Rotor speed, 59,780 r.p.m. Rotor temperature, 24.4 C.

FIG. 2. Electrophoretic patterns of *R. vernicifera* blue protein in 0.05 *M* phosphate buffer of pH 8.0. Protein concentration, 0.50 g./100 ml. Current, 5.0 mA using a cell with a section area of 0.3 cm^2. Temperature, 15–17°C. Time of electrophoresis, 50 min. Position of the initial boundaries is indicated by the arrows.

Absorption Spectra—The blue protein was strongly blue-colored, but the color was reversibly and completely lost when reduced by the addition of L-ascorbic acid. Fig. 3 shows the absorption spectra of purified blue protein measured by a Hitachi model UCA-1 spectrophotometer.

As can be seen, the absorption spectrum due to the oxidized blue protein was rather unusual. It has three absorption maxima in the visible and near infra-red region; a high peak at 608 mμ, a lower one at 450 mμ, and a low broad peak at about 850 mμ. In the ultraviolet region only a sharp peak at 280 mμ was observed. All of the three absorption peaks in the visible region did not undergo any change upon evacuation and equilibration for 24 hours with pure nitrogen, but all of them reversibly disappeared by the addition of L-ascorbic acid. The ratios of the optical density at 280 mμ to that at 450 mμ and 608 mμ were $OD^{280}/OD^{450}=24.6$ and $OD^{280}/OD^{608}=5.93$, respectively.

Assuming that the three aborption peaks in the visible region are all due to the presence of copper in the blue protein, molar extinction coefficients of the protein per atom of copper were calculated at their absorption maxima. The values obtained were 9.70×10^2/cm./atom Cu/liter at 450 mμ, 403×10^3/cm./atom Cu/liter at 608 mμ, and 7.0×10^2/cm./atom Cu/liter at 850 mμ.

When the solution of the blue protein was heated above 80°C, the blue color disappeared irreversibly, though the protein did not precipitate from the solution. The blue color also disappeared by the addition of strong acids.

Oxidation and Reduction of Blue Protein—The solution of the blue protein in 0.1 *M* phosphate buffer of pH 6.5 was rapidly reduced by L-ascorbic acid, but was not detectably reduced by hydroquinone, catechol, and potassium ferrocyanide. Thus, the oxidation-reduction potential of the blue protein must be far lower than that of *R. vernicifera* laccase which has been measured by Nakamura (*3*) to be +415 mV at 25°C and pH 7.0.

The ascorbate-reduced blue protein was

rapidly re-oxidized by potassium ferricyanide, but the re-oxidation by atmospheric oxygen was very slow. Therefore the protein has no oxidase activity for any substrates. The co-existence of the blue protein with laccase in lacquer latex suggested the possibility that the reduced blue protein may be rapidly re-oxidized by laccase; but the results obtained were quite negative. The very slow re-oxidation of reduced blue protein by air was not accelerated by the addition of *R. vernicifera* laccase, nor was the oxidation of L-ascorbic acid catalyzed by laccase accelerated by the addition of blue protein (Table I).

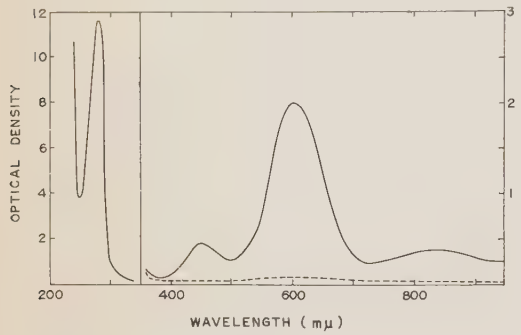

Fig. 3. Absorption spectra of *R. vernicifera* blue protein. 1.00% solution in distilled water. Optical path, 1 cm.
——Oxidized
----Reduced with ascorbic acid

Table I

Effect of the Addition of R. vernicifera Blue Protein on the Oxidation of L-Ascorbic Acid Catalyzed by R. vernicifera Laccase

Additions	O_2-uptake per hour per flask[1]
None	4 μl.
Blue protein (0.90 mg.)	14 〃
〃 +Laccase (0.13 mg.)	188 〃
Laccase (0.13 mg.)	192 〃

1) The reaction mixture per flask consisted of 2 ml. of 0.1 *M* phosphate buffer of pH 6.5, and 1 ml. of 0.05 *M* sodium L-ascorbate solution. Temperature, 25°C.

When a solution of the blue protein was titrated with a dilute solution of L-ascorbic acid, the blue color was found to fade stepwise corresponding to the amount of ascorbic acid added. As can be seen from Fig. 4 and Table II, it was further confirmed by quantitative experiments (conducted with the use of a Cary model 14 recording spectrophotometer) that the extents of decrease in optical density at the three absorption maxima in the visible region were nearly the same at each step of titration. Similar results were also obtained when the reduced blue protein was re-oxidized with ferricyanide. These findings strongly support the view that the absorption peaks are caused by a single component capable of reversible oxidation and reduction and that the purified preparation of blue protein is not a mixture of three colored components each having one absorption maximum in the visible region.

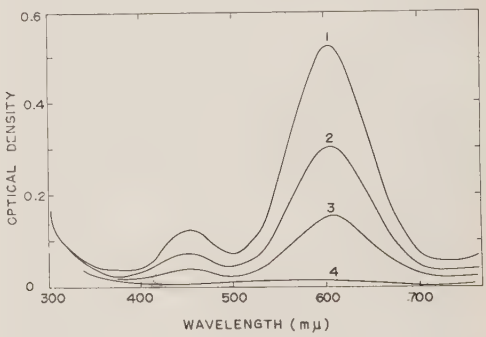

Fig. 4. Stepwise reduction of *R. vernicifera* blue protein with L-ascorbic acid. The optical densities of Curve 2, 3, and 4 were corrected for the dilution caused by the addition of L-ascorbic acid solution.

Curve No.	0.250% blue Solution of protein in 0.1 *M* phosphate buffer of pH 7.0	1 m*M* sodium L-ascorbate solution
1	3.5 ml.	0 ml.
2	3.5 ml.	0.2 ml.
3	3.5 ml.	0.4 ml.
4	3.5 ml.	1.0 ml.

Chemical Properties—R. vernicifera blue protein, in contrast to the Keilin-Mann blue pigment, was found to contain copper. No metals other than copper could be detected by usual methods of analysis. The copper content of the purified preparation was

TABLE II

Stepwise Reduction of R. vernicifera Blue Protein with L-Ascorbic Acid

Exp. No.	Optical density at 450 mμ	608 mμ	850 mμ
1–1: Oxidized[1)]	0.111 (100%)	0.511 (100%)	—
1–2: Partly reduced	0.070 (61%)	0.311 (63%)	—
1–3: Partly reduced	0.031 (28%)	0.159 (31%)	—
2–1: Oxidized	0.112 (100%)	0.512 (100%)	0.081 (100%)
2–2: Partly reduced	0.044 (39%)	0.191 (37%)	0.027 (33%)

1) Exp. 1–1, 1–2, and 1–3 corresponds to Curve 1, 2, and 3 of Fig. 4, respectively.

determined to be 0.328 per cent on the dry weight basis. This value may be compared with the copper content of *R. vernicifera* laccase (0.255 per cent) (*2*). The copper was firmly bound to the protein; prolonged dialysis against 0.1 *M* acetate buffer of pH 5.5 failed to remove any copper from the blue protein.

The nitrogen content measured by the micro-Kjeldahl method was 11.1 per cent. The relatively low content of nitrogen suggests the presence of non-protein components in the molecule of blue protein. It was, in fact, possible to detect the presence of carbohydrates by the thymol-sulfuric acid method (*4*).

Molecular Weight—Solutions of the purified blue protein in 0.1 *M* phosphate buffer of pH 6.5 were subjected to the measurements of partial specific volume, sedimentation coefficient, and diffusion coefficient.

The partial specific volume was determined by measuring the specific gravity of 0.896% solution at 25°C using an Ostwald-type picnometer, and was found to be 0.68 ml. $g.^{-1}$

Sedimentation measurements were carried out with a Spinco model E analytical ultracentrifuge. Three sedimentation experiments at protein concentrations of 0.27, 0.35, and 0.54 per cent, were performed, and the corrected values of sedimentation coefficient (S_{20w}) obtained were 2.08×10^{-13}, 2.21×10^{-13}, and 2.26×10^{-13} cm. $sec.^{-1}$, respectively. By extrapolating to zero concentration, the sedimentation coefficient of the blue protein was estimated to be 2.0×10^{-13} cm. $sec.^{-1}$

Diffusion measurement was carried out with a Spinco model H electrophoresis apparatus. The corrected diffusion coefficient (D_{20w}) of the blue protein was determined to be 6.0×10^{-7} cm^2. $sec.^{-1}$ at a protein concentration of 0.36 per cent.

Using these values, the molecular weight (M_{SD}) of *R. vernicifera* blue protein was computed to be 2.5×10^4. The minimum molecular weight of blue protein was estimated from the content of copper (0.328 per cent) to be 2.0×10^4. It may be concluded that *R. vernicifera* blue protein contain 1 atom of copper per molecule.

DISCUSSION

Rhus vernicifera blue protein, physical and chemical properties of which are described in the present paper, is not identical with the blue pigment reported by Keilin and Mann (*1*). The blue-colored component purified in the present study is a copper-protein lacking oxidase activity. It can be sharply separated from laccase by ion-exchange chromatography, and is a component originally contained in the lacquer latex. According to the report of Keilin and Mann, their blue pigment was isolated from highly purified preparations of lacquer tree laccase, and was free of copper and protein. In this laboratory it was impossible to separate such blue pigment from the purified preparations of *Rhus vernicifera* laccase prepared by ion-exchange chromatography.

The possibility that the blue protein purified in the present study may be a denatured product of laccase should be carefully considered. As the latex employed in this study had been left at room temperature for at least several weeks from the time of collection of freshly secreted latex, it is likely that many of the proteins and enzymes orginally present in the fresh latex may have undergone denaturation during the storage. The content of active laccase in the latex indeed decreases by the long storage of latex at room temperature, and even purified laccase loses gradually its activity in solution stored in a refrigerator. It was, however, found that the inactivated laccase was not identical with the blue protein. The inactivated laccase obtained by months of storage of the purified enzyme solution in a refrigerator still contained nearly the same amounts of firmly-bound copper, and showed the same sedimentation coefficient as those of native laccase. The dissociation of native laccase into fragments with lower sedimentation coefficient by denaturation was not observed. Moreover, the content of blue protein in latex was not increased by the long storage of lacquer latex at room temperature. The blue protein must have been present in lacquer latex from the time of secretion by the wood tissues.

The three-banded spectrum of the oxidized blue protein in the visible and near infra-red region is rather peculiar; none of the known copper-proteins possess such a complex spectrum*. It was, therefore, feared that the purified preparation was still a mixture of two or more components. Such a possibility, however, seems to be excluded from the homogeneity of the preparation in ultracentrifugation and electrophoresis, its failure in separating into colored components on the resin column, and the data on its spectrophotometric titration with L-ascorbic acid.

The blue protein can be reduced by L-ascorbic acid and rapidly re-oxidized by ferricyanide, but the re-oxidation by atmospheric oxygen is extremely slow. Furthermore, the aerobic oxidation of the reduced blue protein cannot be stimulated by the purified laccase. The physiological function of the blue protein is, therefore, quite obscure at present. It appears probable, however, that the blue protein is functioning in the fresh latex as an intermediary electron carrier between certain reducing system and an unknown oxidase. Another possibility is that it is involved in the biosynthesis of laccase in the lacquer tree. These possibilities are to be investigated in future.

* Recently, S. Kato reported at the 33rd General Meeting of the Japanese Biochemical Society, Nov., 1960, that the oxidized form of plastocyanin, a copper-protein obtained from green leaves of many kinds of plants, was found to have a three-banded absorption spectrum similar to that of *Rhus vernicifera* blue protein. This is another example of copper-proteins having multi-banded absorption spectrum in the visible region.

SUMMARY

1. A new blue protein was isolated from the latex of Japanese laquer tree (*Rhus vernicifera*), and purified by column chromatography on an ion-exchange resin. The purified preparation was electrophoretically and ultracentrifugically homogeneous.

2. The blue protein was found to contain 1 atom of firmly bound copper per molecule. The absorption spectrum of the oxidized from has three absorption maxima (850, 608, and 450 mμ) in the visible and near infra-red region, and a maximum (280 mμ) in the ultraviolet region. The three absorption peaks in the visible and near infra-red region disappeared on reduction.

3. The blue protein could be reversibly reduced and oxidized by L-ascorbic acid and potassium ferricyanide respectively. The autoxidation of the reduced form by atmospheric oxygen was, however, very slow indicating the lack of oxidase activity.

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the Institute for Infectious Diseases, University of Tokyo, for sedimentation measurements, to Miss K. Watatsu the Institute of Applied Microbiology, University of Tokyo, for the operation of Spinco model H electrophoresis apparatus, and to Miss K. Ichino for her co-operation in this work. The author also expresses his gratitude to Saito Co. Ltd. for the kind supply of lacquer latexes.

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Hemocyanin of *Ommatostrephes sloani pacificus*

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Hemocyanin is a copper-containing respiratory protein occurring in the blood of many molluscs and arthropods, and numerous investigations have been reported on the nature of the hemocyanins obtained from various species of animals (*1*). Especially, hemocyanins of snails, crabs, and lobsters were the subject of many studies. However, relatively little information is available concerning the properties of hemocyanins from cephalopods.

In the present study, the hemocyanin of *Ommatostrephes sloani pacificus*, a common species of medium-sized squid, was purified by an isoelectric precipitation procedure. The purification procedure and the properties of the purified hemocyanin will be described in this paper.

EXPERIMENTALS AND RESULTS

Purification of the Hemocyanin from the Blood of Ommatostrephes sloani pacificus—The blood was withdrawn from the vascular system of living squids by means of a hypodermic syringe. The blood, which had been almost colorless in the vascular system of living animals, turned into deep blue upon the contact with air, but showed no tendency of clotting. About 3 ml. of the blood was usually obtained from one animal with average body weight of 300 g. All stages of the following purification procedures were carried out in the cold, and glass-distilled water was throughout used.

The blood was centrifuged at 10,000 r.p.m. for 20 minutes, and the white fibrous precipitate was discarded. The clear, deeply blue-colored supernatant was then dialyzed against distilled water for 24 hours with 2 or 3 changes of the external fluid. Small amounts of white precipitate formed during the dialysis were centrifuged off and discarded. The dialyzed solution obtained from 100 ml. of the blood was diluted with 1 liter of 0.02 *M* phosphate buffer, pH 8.0. The diluted solution was then gradually acidified by the dropwise addition of 0.1 *M* acetate buffer of pH 4.5 with constant gentle stirring. When the pH of the solution was lowered to 5.0 the addition of acetate buffer was stopped, and the solution was left for several hours with occasional stirring. As the solubility of the hemocyanin of this squid is very low at around pH 5.0 (Fig. 1), almost all hemocyanin contained in the dialyzed solution was precipitated in fine crystalline form.

The precipitated hemocyanin was collected by centrifugation, dissolved in 500 ml. of 0.02 *M* phosphate buffer of pH 8.0, and a small amount of insoluble materials were removed by centrifugation. The hemocyanin was again precipitated from the solution by adding 0.1 *M* acetate buffer of pH 4.5 and adjusting the pH of the solution to about 5.0. This procedure of isoelectric precipitation was repeated once more, and the final precipitate of purified hemocyanin was dissolved in 200 ml. of 0.02 *M* phosphate buffer, pH 8.0. The solution was then dialyzed against distilled water. Usually 12 to 14 g. of purified hemocyanin were thus obtained from 100 ml. of blood.

The solution of purified hemocyanin obtained by prolonged dialysis against distilled water was purplish blue, the visible absorption

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spectrum of which having a broad peak in the red region with a maximum at about 580 mμ. In the ultraviolet region, two strong absorption peaks at 280 and 347 mμ were observed, and the latter of the two peaks and the broad peak in the red region disappeared on deoxygenation (Fig. 2).

The solution of hemocyanin could be stored in a refrigerator for weeks without any change in its physico-chemical properties. The dialyzed solution was susceptible to surface denaturation, and the insoluble denatured protein formed by shaking the solution was no longer soluble even in salt solutions; the solutions containing buffer salts were more resistant against surface denaturation.

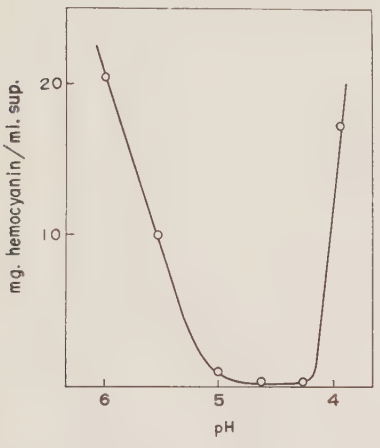

Fig. 1. Solubility of hemocyanin in 0.1 *M* acetate buffer. Dialyzed stock solution of the purified hemocyanin was properly diluted with 0.1 *M* acetate buffer of various pH values, and the solutions were kept at 4°C for ten hours with stirring. The solutions were then centrifuged to to remove precipitated excess hemocyanin. The concentration of hemocyanin in the supernatants was determined by micro-Kjeldahl nitrogen analysis.

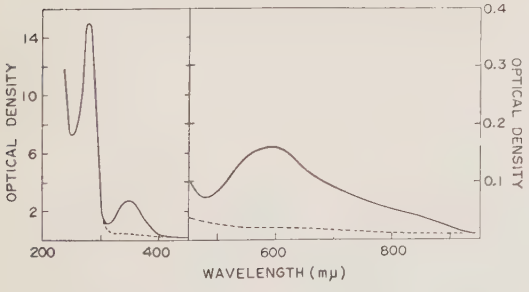

Fig. 2. Absorption spectra of purified hemocyanin. 1.00 % solution in 0.1 *M* phosphate buffer of pH 7.5. Optical path, 1 cm.
— Oxygenated
..... Deoxygenated

Association and Dissociation of the Purified Hemocyanin—The dialyzed solution of the purified hemocyanin was diluted with 0.1 *M* acetate (pH 3.5–6.0), 0.1 *M* phosphate (pH 6.0–8.5), or 0.1 *M* ammonia hydrochloric acid (pH 8.5–10.0) buffers of varying pH values, and the solutions were kept at 4°C for 24 hours to ensure the at tainment of equilibrium. Then the solutions were studied by sedimentation and electrophoresis analyses.

Both methods showed that the hemocyanin was composed of only one component at pH's between 7.0 and 8.5, indicating the homogeneity of the purified preparation (Figs. 3, 4). At the acidic side of pH 7.0 an associated component with higher sedimentation coefficient and larger electrophoretic mobility appeared, and the associated component increased with increasing acidity (Figs. 3, 4). The association and dissociation were completely reversible between pH 8.5 and 5.6, though the velocity of these reactions seemed to be relatively slow and at least several hours were needed to ensure the attainment of equilibrium between the dissociated and associated components. At pH's between 5.6 and 4.2, the sedimentation and the electrophoresis analyses were impossible owing to the low solubility of the protein. At higher pH's than 8.5, the dissociation became partly irreversible.

Molecular Weight of the Dissociated Component—The molecular weight of the dissociated component was determined by measuring its sedimentation coefficient, diffusion coefficient, and partial specific volume, and substituting these values in the standard equation for molecular weight (*2*). Solutions of the purified hemocyanin in 0.1 *M* phosphate buffer of pH 7.5 were used in all determinations. In the case of dry weight determinations, the

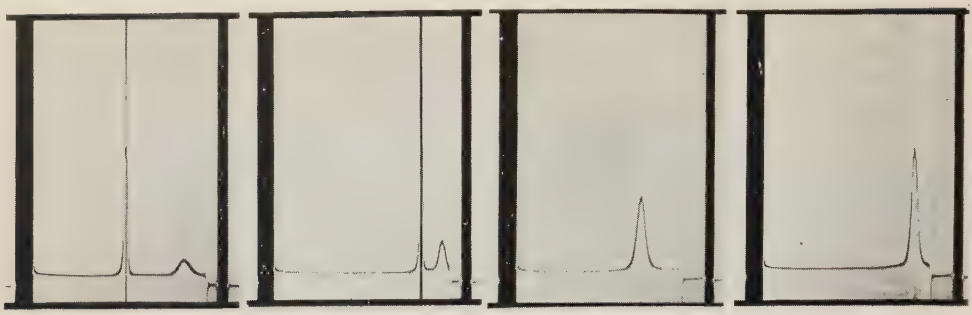

Fig. 3. Sedimentation patterns of purified hemocyanin in 0.1 *M* phosphate buffer. A Spinco model E analytical ultracentrifuge was used. pH's of the media are 7.5 (right) and 5.8 (left). Protein concentration, 0.77 g./100 ml. Rotor speed, 31,410 r. p. m. Rotor temperature, 15.0°C (right) and 26.6°C (left).

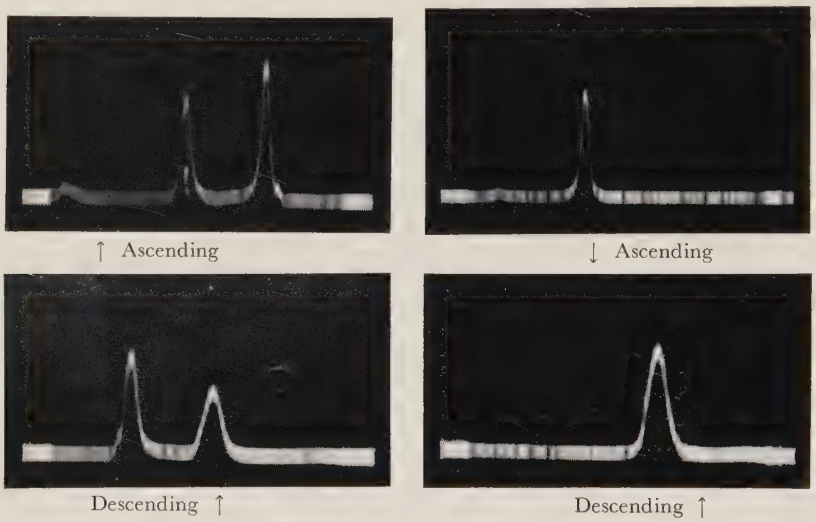

Fig. 4. Electrophoresis patterns of purified hemocyanin in 0.05 *M* phosphate buffer. A Hitachi model HTB Tiselius-type apparatus was used. pH's of the media are 7.5 (right) and 6.2 (left). Protein concentration, 1.50 g./100 ml. Current, 8.0 mA using a cell with a section area of 0.3 cm^2. Temperature, 12-13°C. Time of electrophoresis, 40 minutes. Position of the initial boundaries is indicated by the arrows.

dialyzed samples were dried at 110°C in air to constant weight.

The sedimentation measurements were carried out using a Spinco model E analytical ulturacentrifuge. Two solutions of hemocyanin, the protein concentrations of which were 0.360 and 0.720 per cent respectively, were subjected to the sedimentation analysis, and the sedimentation coefficients obtained were corrected to an average rotor temperature at 20°C in water. By extrapolating these values to zero concentration, S_{20w} was estimated to be 19.5×10^{-13} cm. sec^{-1}.

The diffusion coefficient was determined at 4°C with 0.358 and 0.716 per cent solutions using a Spinco model H electrophoresis apparatus. The diffusion coefficients, calculated by the maximal ordinate—area method, were corrected to the conditions prevailing in water at 20°C, and then the two values obtained at two protein concentrations, were extrapolated to zero concentration, giving a D_{20w} of 2.80×10^{-7} cm^2. sec^{-1}.

The partial specific volume was determined by measuring the densities of the hemocyanin solutions of 1.59 and 3.18 per cent

concentrations using an Ostwald-type picnometer. The value obtained was 0.724 ml. g^{-1}. at 20°C.

From the above quantities the molecular weight of the dissociated component was calculated to be 6.13×10^5. As the minimal molecular weight of the hemocyanin calculated from the content of copper, 0.260 per cent, is 2.45×10^4, it may be concluded that one molecule of the dissociated component contains about 25 atoms of copper.

Metals in the Purified Hemocyanin—The metal components of the purified hemocyanin were analyzed by the emission spectrographic method according to the previously described procedures (*3*). Besides copper, the main metal component of the protein, calcium, magnesium, and a trace of silver were always detected. The content of copper was determined by the dithizone method (*3*), and found to be 0.260 per cent.

Calcium and magnesium could be removed from the hemocyanin molecule by dialysis against 0.005 *M* ethylenediamine tetracetate solution, and the ethylenediamine tetracetate-dialyzed preparations showed the same association-dissociation properties as the non-treated samples.

As mentioned above, traces of silver could invariably be detected in all the purified preparations when analyzed by the emission spectrography; two sensitive emission lines of silver (3280.68 and 3382.89 Å) were observed. The possibility of the contamination of silver during the analytical procedures was carefully investigated and excluded. The content of silver in the hemocyanin, however, was very low, the content determined by the dithizone method (*4*) being less than 0.001 per cent. The silver could be completely removed by dialysis against 0.01 *M* KCN solution.

DISCUSSION

Hemocyanin of *Ommatostrephes sloani pacificus* was easily purified from the blood by an isoelectric precipitation procedure. The purified preparation was electrophoretically and ultracentrifugically homogenous, and very stable in solution, though freeze-drying was found to cause considerable denaturation of the protein as already described by Litt and Boyd (*5*) on hemocyanins of *Busycon canaliculatum* and *Limulus polyphemus*. As the species of squid employed in this study is readily obtainable in quantities all the year round, the hemocyanin of this animal may be a convenient material for further investigations on the chemical nature of hemocyanin.

The presence of calcium and magnesium in the purified hemocyanin is of interest, for these metals have been reported to prevent the dissociation of *Helix pomatia* hemocyanin, and to protect the hemocyanin molecule from the irreversible dissociation in alkaline solutions (*6*). In the present study, however, the pH-dependent reversible equilibrium between the associated and the dissociated components was not affected by the presence of ethylenediamine tetracetate which could remove these metals from the hemocyanin molecule.

The detection of traces of silver in the purified preparations of hemocyanin was rather unexpected. Silver has often been found to be a constituent of certain marine invertebrates (*7*), but its concentrations were too low to conclude any significant accumulation of the element from the environments. Furthermore, no information is as yet available on the nature of the compounds associated with the presence of silver in such biological materials. The finding that purified hemocyanin contains silver may account for the occurrence of the element in marine molluscs and arthropods.

SUMMARY

1. An isoelectric precipitation procedure for the purification of hemocyanin from the blood of *Ommatostrephes sloani pacificus* was described.

2. Purified hemocyanin obtained by the procedure was electrophoretically and ultracentrifugically homogenous at pH's between 7.0 and 8.5 in 0.1 *M* phosphate buffer. In more acidic media, an associated component was reversibly formed in an equilibrium with the dissociated form.

3. The molecular weight of the dissociated component was 6.13×10^5.

4. Purified hemocyanin contained 0.260% copper, and traces of silver were found to be always present.

The authors wish to express their hearty gratitude to the late Prof. A. Nishimura for his interest and encouragement during this investigation, and to Prof. Y. Ogura, University of Tokyo, for his valuable advices. Thanks are also due to Miss K. Watatsu, the institute of Applied Microbiology, University of Tokyo, for the operation of Spinco model H electrophoresis apparatus. The authors also express their gratitude to Messrs. K. Mori, H. Hiyoshi, and M. Kawaguchi, the Fisheries Experimental Station of Shizuoka-ken, for their kind aid in obtaining fresh-caught, living squids.

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Research on Pancreatic Ribonuclease

I. The Inhibition of Cyclic Phosphodiesterase Activity of Bovine Pancreatic Ribonuclease by Several Substrate Analogues

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In the resent years a great deal of interest has been shown by a number of researchers in the mechanism of enzymatic reaction of bovine pancreatic ribonuclease (RNase-IA) at the molecular level. In particular, these studies on the RNase-IA have elucidated the enzyme's entire sequence of covalent structure (*1*), disclosed some of its essential and unessential residues (*2–6*) and made an approach to a three-dimensional model of this enzyme. (*7*).

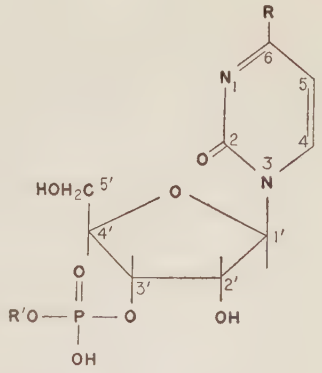
These findings, therefore, have added an impetus to the further research on substrate specificity of the enzyme which is generally known to possess both transphosphorylase and cyclic phosphodiesterase activities.

Witzel (*8*, *9*) proposed that the specific structure of the substrate for RNase-IA activity was a phosphodiester of 3′-ribonucleotide which possesses a C_6-hydroxy or amino-substituted 2-pyrimidinone residue linked by a β-ribonucleosidic bond* at its N_3 position.

This structure is represented in the diagram in the next column.

Witzel, also proposed that the mechanism for the enzyme-substrate reaction involved a concerted reaction occurring in the substrate molecule bound on the active site of the enzyme surface.

While there were numerous reports on the structural specificity of various nucleotides as a substrate for RNase-IA, very little information could be found on the inhibition effects on this enzyme by analogues of these substrate compounds. Some other studies (*11–13*) showed that several anionic polymers, such as heparin and polyxenylphosphate, were found to inhibit the activity of the RNase but their reaction mechanisms were not clearly described. Also, it is commonly known that the accumulation of the final products of RNase reaction, pyrimidine 3′-ribonucleotides, decreases the rate of hydrolysis reaction of ribonucleic acid and that 2′-analogues of the ribonucleotide or several other mononucleotides also inhibit the cyclic phosphodiesterase activity of the enzyme (*11*, *14–16*).



R = OH or NH_2
R′ = nucleoside or suitable alkyl residue

In these circumstances, it seemed interesting to investigate the rate and mode of inhibition of various compounds having a structure totally or partially analogous to those of the nucleotides which serve as substrates for the RNase reaction. If these test

* 2′ : 3′-Cyclic pseudouridylic acid was also found to be an active substrate for this enzyme (Davis, F. F., and Allen, F. W., *J. Biol. Chem.*, **227**, 907 (1957)).

compounds inhibit RNase reaction competitively, the results obtained in this research should afford valuable informations on the mode and site of affinity of the substrate to the enzyme.

Therefore, various derivatives of 2(3H)-pyrimidinone (2-hydroxypyrimidine) and their nucleosides and nucleotides and some other phosphorous compounds were tested as to their inhibitory property on cyclic phosphodiesterase activity of RNase-IA using cytidine or uridine 2′:3′-cyclic phosphate as a substrate. The methods used and the results obtained in this study of various possible inhibitors of RNase-IA activity were reported in this paper.

EXPERIMENTAL

Material Used as Inhibitor—Some of the compounds used as inhibitors were synthesized by methods described in the cited references and the others were kindly given to us by other researchers or were purchased from the firms whose names are cited in parentheses. 2(3H)-pyrimidinone (No. 1) (*17*), uracil (No. 2), thymine (No. 3) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), 5-bromouracil (No. 4) (*18*), 5-aminouracil (No. 5) (*19*), 4, 5-dihydrouracil (No. 7) (*20*), 3-methyluracil (No. 8) (*21*), 2-thiouracil (No. 9), cytosine (No. 10) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), 3-methylcytosine (No. 11) (*22*), 1, 3-dimethyluracil (No. 12) (*23*), 1-methyluracil (No. 13) (*21*), 2, 3-dihydro-6-ethoxy-2-oxopyrimidine (No. 14) (*24*), 3, 6-dihydro-2-ethoxy-6-oxopyrimidine (No. 15) (*24*), 5-cyanouracil (No. 16), 5-aminomethyluracil (No. 17), 5-dimethylaminomethyluracil (No. 18), 3-[(1, 2, 3, 4-tetrahydro-2,4-dioxo-5-pyrimidinyl)-methyl]-4-methyl-5-(2-hydroxyethyl)-thiazolium nitrate (No. 21) (Prof. S. Yamada of this Faculty), barbituric acid (No. 19), 4(3H)-pyrimidinone (No. 20) (*25*), uridine (No. 22) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), thymine riboside (No. 23) (*26*), thymidine (No. 24) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), thymine xylofuranoside (No. 25) (*26*), thymine glucopyranoside (No. 26) (*26*), 5-bromouridine (No. 27), (*27*), cytidine (No. 28), deoxycytidine (No. 29) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), 5-morpholinouridine (No. 30) (Prof. Y. Mizuno of the Hokkaido University), adenosine (No. 31) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), inosine (No. 32) (*28*), benzimidazole riboside (No. 33) (Prof. D. Mizuno of this Faculty), 6-deoxyuridine (No. 34) (*29*), 2, 6-dideoxy-4-oxouridine (No. 35) (*29*), benzyl riboside (No. 36) (*30*) adenosine 2′ (+3′)-phosphate (No. 38), adenosine 5′-phosphate (No. 39) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), adenosine 2′:3′-cyclic phosphate (No. 40), adenosine 5′-triphosphate (No. 41) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), guanosine 2′(+3′)-phosphate (No. 42) (*31*), guanosine 5′-phosphate (No. 43) (Pabst Laboratories, Milwaukee, Wiss.), guanosine 2′:3′-cyclic phosphate (No. 44), inosine 2′(+3′)-phosphate (No. 45) (*31*), inosine 5′-phosphate (No. 46) (*31*), uridine 2′(+3′)-phosphate (No. 47), uridine 5′-phosphate (No. 48) (Pabst Laboratories, Milwaukee, Wiss.), 2′,3′-isopropylideneuridine 5′-phosphate (No. 49)*, 5-bromouridine 2′(+3′)-phosphate (No. 50) (*32*), 5-bromouridine 5′-phosphate (No. 51) (Prof. Y. Mizuno of the Hokkaido University), cytidine 2′(+3′)-phosphate (No. 52) (Schwarz Bioresearch, Inc. Mount Vernon, N. Y.), cytidine 5′-phosphate (No. 53) (Pabst Laboratories, Milwaukee, Wiss.), ribothymidine 5′-phosphate (No. 54)**, thymine-N_3-β-D-glucoside 6′-phosphate (No. 55)***, methyl riboside 2 (+3)-phosphate (No. 56) (*33*), phenyl phosphate (No. 58), diphenyl phosphate (No. 59), ethylene glycol cyclic phosphate (No. 60) (*34*), ethylene glycol monophosphate (No. 61) (*34*), hydrobenzoin phosphate (No. 62) (*35*), hydrobenzoin cyclic phosphate (No. 63), (*35*).

Ribonuclease-IA—RNase-I was extracted from bovine pancreas in a crystalline form according to the procedure of Kunitz (*36*) and then further fractionated by the ion exchange column chromatography method of Moore and Stein (*37*) using Amberlite IRC-50 to isolate the main component, RNase-IA. The combined fractions containing the RNase-IA were dialyzed and then lyophilized. A reproducible single peak could be obtained for this purified enzyme preparation when it was subjected again to ion-exchange chromatography. This preparation was then proved to contain no phosphodiesterase activity using bis-*p*-nitrophenyl phosphate as a substrate in the method outlined by Yoshida (*38*). It was then assayed and submitted to various inhibition tests.

Assay of Enzyme Activity—The activity of the ribonuclease preparation was estimated according to a modified procedure of Zittle (*39*) using Warburg manometers. 0.5 ml. of enzyme solution containing 33 μg. of RNase-IA was placed into the side arm. 1.0 ml. of aqueous solution containing 30.8 μmol. of ammonium 2′:3′-cyclic cytidylate or uridylate, 1.0 ml. of 0.1 *N* sodium bicarbonate solution and a inhibitor compound dissolved in 1.0 ml. of water were introduced successively into the main compartment of each

* T. Ukita, N. Imura and K. Nagasawa, unpublished.

, * T. Ukita and H. Hayatsu, unpublished.

flask and the system was thoroughly equilibrated with a mixture of nitrogen and carbon dioxide (95 : 5). After equilibrium was reached, the enzyme solution in the side arm was allowed to flow into the flask and the manometer for each reaction was read at five-minute intervals. The pH attained by this system was 7.6 and the temperature was kept constant at 37°C. As is shown in Fig. 1, the rate of CO_2 evolution was found linear to the function of time during the first 20 minutes.

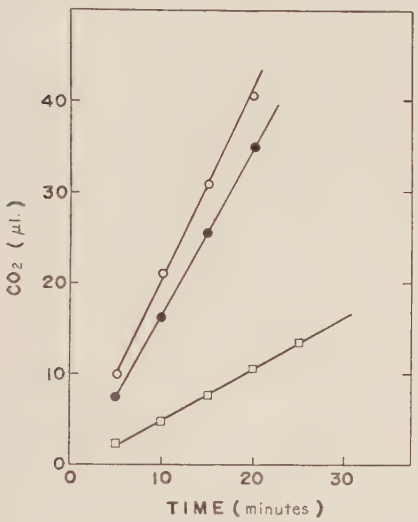


FIG. 1. Hydrolysis of ammonium cytidine 2′ : 3′-cyclic phosphate by RNase-IA, with and without inhibitor. —○— control, —●— 2-thiouracil added, —□— ATP added

Unless otherwise noted, the degree of inhibition for a compound is represented by an inhibition index which is the decrease in percent of the tangent of slope from that of the control when equimolar amounts of inhibitor and substrate were used. As for the control reaction the same system was used except that the solution of inhibitor was replaced by a sodium chloride solution of the same ionic strength. In these experiments, no retention of CO_2 was observed both in the control tests and in the test in which inorganic phosphate was used as an inhibitor when checked by the method of Bain and Rush (*40*).

RESULTS

As is shown in Table I, those derivatives of 2(3H)-pyrimidinone, which could be converted to active substrates for RNase-IA when their N_3-positions were substituted with a suitable phosphodiester of 3′-phosphoryl ribofuranosyl group, generally interfered with the hydrolysis of 2′ : 3′-cyclic cytidylic acid by RNase-IA. However, cytosine (No. 10) did not show any inhibitory activity even when four times the molar amount of the compound to that of the substrate was used.

The five derivatives of 2(3H)-pyrimidinone consisting of 5-nitrouracil (No. 6), 5-cyanouracil (No. 16), 5-aminomethyl- (No. 17), 5-dimethylaminomethyluracil (No. 18) and barbituric acid (No. 19), none of whose 3-ribofuranosyl 3′-phosphodiesters has ever been synthesized, also exhibited a pronounced inhibition in this reaction system. The inhibitory reactions caused by compounds Nos. 6, 17 and 18 were much more pronounced than that by uracil. In contrast to these reactions, compound No. 21 which contains a bulky substitution on the β-nitrogen of the methylamino group did not show any inhibitory activity.

The structural relationship of compounds Nos. 1, 20, 14 and 15 is that one of the two hydroxyl groups in the uracil ring is substituted with hydrogen or ethoxyl group. This group of compounds did not interfere with the enzymatic hydrolysis when equimolar amounts of the compounds were added to the reaction mixture. Among the three N-methylated uracils, compounds Nos. 8, 12 and 13, 1, 3-dimethyl uracil (No. 12) which has no enolizable hydrogen on two nitrogens in the pyrimidine ring did not reveal any inhibition effect. 1-Methyl uracil (No. 13) showed some inhibition only at a high concentration of the compound while 3-methyl uracil (No. 8) inhibited the reaction in the same degree as did uracil.

Table II shows the similar inhibitory property of several N_3-glycosides of 2(3H)-pyrimidinone derivatives.

Several nucleosides were studied for their inhibitory effects on RNase-IA activity. The naturally occurring pyrimidine ribo- and deoxyribonucleosides and 5-bromo and 5-morpholino substituted uridine exhibited an inhibitory property for the hydrolytic cleavage of 2′ : 3′-cyclic cytidylic acid by RNase-IA. It is interesting to note that the relative activity

of these inhibitions is greater than those exhibited by the parent 2(3H)-pyrimidinones corresponding to each N_3-glycosides when equimolar amounts of inhibitor and substrate are used. No inhibition was effected by naturally occurring purine ribofuranosides, benzimidazole-N_9-β-D-ribofuranoside (No. 33), 1-O-benzyl-β-D-ribofuranoside (No. 36) and D-ribose (No. 37). Both 6-deoxyuridine (No. 34) and 2, 6-dideoxy-4-oxo-uridine (No. 35) did not inhibit the enzymatic hydrolysis of the substrate but a larger amount of the former showed some inhibition.

The inhibitory activity of thymine ribofuranoside (No. 23) and cytidine (No. 28) was diminished when the ribofuranosyl moiety was replaced with a deoxyribofuranosyl group.

The conversion in the configuration of the hydroxyl group at C_3' position of thymine ribofuranoside did not cause any decrease in the inhibitory activity thus thymine xylofuranoside (No. 25) exhibited an appreciable increase. On the other hand, the substitution of a glucopyanosyl group at N_3-position of thymine reversed the inhibitory property of the parent base, thymine. Thus thymine glucopyranoside (No. 26) revealed virtually no inhibition.

The inhibitory properties of several ribofuranotides are summarized in Table III. All the nucleotides tested which contained secondary phosphate dissociation in their phosphoryl groups exhibited a remarkably strong inhibitory property irrespective of its base moiety,

Table I

Inhibition by Several 2(3H)-Pyrimidinones on the Hydrolysis of Ammonium 2′ : 3′-Cyclic Cytidylate by RNase-IA

No.	Inhibitor; Derivatives of 2(3H)-Pyrimidinone (2-hydroxypyrimidine)		Inhibition	Index
1.	Non substituted		0	31[b]
2.	6-Hydroxy-	(Uracil)	24	48[a]
3.	5-Methyl-6-hydroxy-	(Thymine)	26	
4.	5-Bromo-6-hydroxy-	(5-Bromouracil)	23	
5.	5-Amino-6-hydroxy-	(5-Aminouracil)	0	
6.	5-Nitro-6-hydroxy-	(5-Nitrouracil)	55	
7.	4, 5-Dihydro-6-hydroxy-	(4, 5-Dihydrouracil)	17	
8.	3-Methyl-6-hydroxy-	(3-Methyluracil)	18	
9.	2-Thio-6-hydroxy-	(2-Thiouracil)	13	
10.	6-Amino-	(Cytosine)	0	0[b]
11.	3-Methyl-6-amino-	(3-Methylcytosine)	0	
12.	1, 6-Dihydro-1, 3-dimethyl-6-oxo-	(1, 3-Dimethyluracil)	0	0[b]
13.	1, 6-Dihydro-1-methyl-6-oxo-	(1-Methyluracil)	0	17[b]
14.	2, 3-Dihydro-6-ethoxy-		0	19[b]
15.	3, 6-Dihydro-2-O-ethyl-6-oxo-		0	34[b]
16.	5-Cyano-6-hydroxy-	(5-Cyanouracil)	26	
17.	5-Aminomethyl-6-hydroxy-	(5-Aminomethyluracil)	53	
18.	5-Dimethylaminomethyl-6-hydroxy-	(5-Dimethylaminomethyluracil)	40	
19.	1, 6-Dihydro-4-hydroxy-6-oxo-	(Barbituric acid)	35	
	Inhitor; Other than 2(3H)-Pyrimidinone derivatives			
20.	4(3H)-Pyrimidinone		0	39[b]
21.	3-[(1,2,3,4-Tetrahydro-2,4-dioxo-5-pyrimidinyl)-methyl] -4-methyl-5-(2-hydroxyethyl)-thiazolium nitrate		2	

a) The amount of the inhibitor added was two molar equivalents to the amount of substrate used.
b) The amount of the inhibitor added was four molar equivalents to the amount of substrate used.

TABLE II

Inhibition by Several Nucleosides on the Hydrolysis of Ammonium 2′ : 3′-Cyclic Cytidylate by RNase-IA

No.	Inhibitor	Inhibition Index	
22.	Uracil-N_3-β-D-ribofuranoside (Uridine)	29	48[a]
23.	ThymineN_3-β-D-ribofuranoside (Thymine riboside)	40	
24.	Thymine-N_3-β-D-deoxyribofuranoside (Thymidine)	32	
25.	Thymine-N_3-β-D-xylofuranoside	85	
26.	Thymine-N_3-β-D-glucopyranoside	4	
27.	5-Bromouracil-N_3-β-D-ribofuranoside (5-Bromouridine)	24	
28.	Cytosine-N_3-β-D-ribofuranoside (Cytidine)	37	
29.	Cytosine-N_3-β-D-deoxyribofuranoside (Deoxycytidine)	19	
30.	5-Morpholinouracil-N_3-β-D-ribofuranoside	18	
31.	Adenine-N_9-β-D-ribofuranoside (Adenosine)	0	
32.	Hypoxanthine-N_9-β-D-ribofuranoside (Inosine)	0	
33.	Benzimidazole-N_9-β-D-ribofuranoside	0	
34.	6-Deoxyuridine	0	20[b]
35.	2, 6-Dideoxy-4-oxouridine	0	0[b]
36.	1-O-Benzyl-β-D-ribofuranoside	0	
37.	D-Ribose	0	0[b]

a) The amount of the inhibitor added was two molar equivalents to the amount of substrate used.
b) The amount of the inhibitor added was four molar equivalents to the amount of substrate used.

whether it was purine, or pyrimidine. The inhibition index was higher for these compounds than those of the corresponding nucleosides which were found to be inhibitory. Furthermore, when pyrimidine ribonucleotides were considered, 5′-phosphate compounds were found generally less inhibitory than those of 2′(+3′)-phosphates of the same nucleoside. Such differences in inhibition indices attributed to the phosphoryl group position in the sugar moiety were not apparent in the case of purine nucleotides. Adenosine- (No. 40) and guanosine-2′ : 3′-cyclic phosphate (No. 44) did not reveal any inhibitory property while adenosine 5′-triphosphate (No. 41), which has four phosphate dissociations, strongly inhibited enzymatic activity. The inhibition by methyl-(α+β)-D-ribofuranoside 2 (+3)-phosphate (No. 56) was much less than that of uridine 2′(+3′)-phosphate (No. 47). Thus, the inhibition indices of pyrimidine-, purine-ribofuranoside 2′(+3′)-phosphates and methyl-ribofuranoside 2(+3)-phosphate fell in this order of magnitude.

The blocking of the 2′- and 3′-hydroxyl groups of uridine 5′-phosphate largely diminished its inhibition index. And the alteration of 5′-phosphoryl ribofuranosyl group of ribothymidine 5′-phosphate (No. 54) with 6′-phosphoryl glucopyranosyl group (No. 55) also reduced the inhibitory activity of the compound.

As is shown in Table III, several phosphoryl ribofuranosides having a secondary phosphate dissociation group could prevent the RNase-IA hydrolysis of 2′ : 3′-cyclic cytidylic acid even though the property of the parent nucleoside was not that of an inhibitor. This demonstrates that the phosphoryl group commonly acts as an inhibitor when it contains a secondary dissociation. The inhibitory property of inorganic phosphate and several organic phosphates on the RNase-IA hydrolysis of 2′ : 3′-cyclic cytidylate was tested and the results are given in Table IV. Both inorganic and organic phosphomono-

Table III

Inhibition by Several Nucleotides on the Hydrolysis of Ammonium 2′ : 3′-Cyclic Cytidylate by RNase-IA

No.	Inhibitor	Inhibition Index
38.	Adenosine 2′(+3′)-phosphate	48
39.	Adenosine 5′-phosphate	44
40.	Adenosine 2′ : 3′-cyclic phosphate	0
41.	Adenosine 5′-triphosphate	71
42.	Guanosine 2′(+3′)-phosphate	64
43.	Guanosine 5′-phosphate	35
44.	Guanosine 2′ : 3′-cyclic phosphate	0
45.	Inosine 2′(+3′)-phosphate	57
46.	Inosine 5′-phosphate	57
47.	Uridine 2′(+3′)-phosphate	80
48.	Uridine 5′-phosphate	54
49.	2′, 3′-Isopropyrideneuridine 5′-phosphate	7
50.	5-Bromouridine 2′(+3′)-phosphate	88
51.	5-Bromouridine 5′-phosphate	58
52.	Cytidine 2′(+3′)-phosphate	82
53.	Cytidine 5′-phosphate	68
54.	Ribothymidine 5′-phosphate	46
55.	Thymine-N_3-β-D-glucoside 6′-phosphate	10
56.	Methyl-($\alpha+\beta$)-D-ribofuranoside 2 (+3)-phosphate	13

Table IV

inhibition by Several Phosphates on the Hydrolysis of Ammonium 2′ : 3′-Cyclic Cytidylate by RNase-IA

No.	Inhibitor	Inhibition Index
57.	Inorganic phosphate	24
58.	Phenyl phosphate	10
59.	Diphenyl phosphate	0
60.	Ethylene glycol cyclic phosphate	−29[a]
61.	Ethylene glycol monophosphate	13
62.	Hydrobenzoin phosphate	20
63.	Hydrobenzoin cyclic phosphate	0

a) The minus sign indicates that the compound accelerated the enzymatic reaction.

esters were found inhibitory while of the three phosphodiesters tested, diphenyl phosphate (No. 59) and hydrobenzoin cyclic phosphate (No. 63) showed no influence on the enzymatic activity. As an exception ethylene glycol cyclic phosphate (No. 60) exhibited a pronounced stimulation of RNase-IA activity.

To fully understand the mode of inhibition of various types of phosphate compounds on RNase-IA hydrolysis of 2′ : 3′-cyclic cytidylic acid, some representative compounds from different groups were examined as to their nature of RNase-IA inhibition by using the

Lineweaver-Burk's method (*41*). The test compounds included uracil (No. 2), uridine (No. 22), cytidine (No. 28), 5-bromouridine (No. 27), thymidine (No. 24), adenosine 2′(+3′)-phosphate (No. 38), adenosine 5′-phosphate (No. 39), adenosine 5′-triphosphate (No. 41), uridine 2′(+3′)-phosphate (No. 47), uridine 5′-phosphate (No. 48), phenyl phosphate (No. 58), and inorganic phosphate (No. 57).

It was revealed that all of the compounds tested were found to show competitive type of inhibition; the result which was obtained for uracil for an example, is shown in Fig. 2. The K*i* values calculated for each inhibitor were summarized in Table V and these values reflected the inhibitory property of those individual compounds.

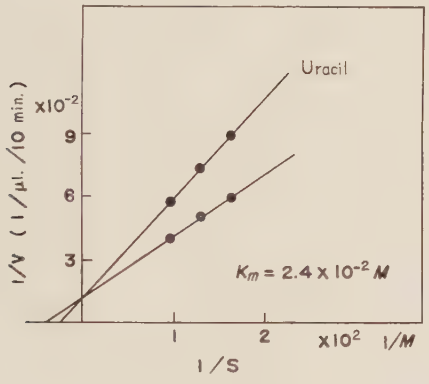


Fig. 2. Lineweaver-Burk plot showing the relationship between cyclic phosphodiesterase activity and substrate concentration in the absence of uracil and in the presence of uracil (in equimolar amount to that of the substrate).

Under the same experimental conditions the inhibitory property of pyrimidinones and their ribofuranosides on the cyclic phosphodiesterase activity of RNase-IA was examined using ammonium 2′:3′-cyclic uridylate as a substrate*. The inhibition indices for these compounds were presented in Table VI. As is shown in the table, cytosine, uracil, cytidine and uridine gave inhibition indices which revealed similar inhibitory property to those observed for these compounds when ammonium 2′:3′-cyclic cytidylate was used as the substrate for the enzyme.

Table V

Ki Values of Several Inhibitors

Uracil	1.8×10^{-2} *M*
Uridine	1.3×10^{-2} //
Cytidine	2.5×10^{-2} //
5-Bromouridine	1.4×10^{-2} //
Thymidine	5.0×10^{-2} //
Adenosine 2′(+3′)-phosphate	3.5×10^{-2} //
Adenosine 5′-phosphate	3.3×10^{-2} //
Adenosine 5′-triphosphate	3.8×10^{-3} //
Uridine 2′(+3′)-phosphate	2.4×10^{-3} //
Uridine 5′-phosphate	4.3×10^{-3} //
Phenyl phosphate	1.1×10^{-1} //
Inorganic phosphate	2.9×10^{-3} //

Table VI

Inhibition by Several Bases and Nucleosides on the Hydrolysis of Ammonium 2′:3′-Cyclic Uridylate by RNase-IA

Inhibitor	Inhibition Index (Equimolar Inhibitor to Substrate)
6-Amino-2(3H)-pyrimidinone (Cytosine)	3
6-Hydroxy-2(3H)-pyrimidinone (Uracil)	13
Cytosine-N_3-β-D-ribofuranoside (Cytidine)	55
Uracil-N_3-β-D-ribofuranoside (Uridine)	23

DISCUSSION

The results obtained by this research revealed that among the tested compounds, all 2(3H)-pyrimidinone derivatives** and their ribonucleosides except cytosine were found to inhibit the cyclic phosphodiesterase activity of RNase-IA. This was true when the molecular structures of these compounds are

* The ratio of velocity in the hydrolysis of 2′:3′-cyclic uridylate and 2′:3′-cyclic cytidylate by RNase-IA was 1:4 under conditions used in this experiment.

** Purine bases from naturally occurring purine nucleotides could not be tested for inhibitory activity because of their poor solubility.

contained as a part of the total structure of known active substrate for this enzymatic reaction. Inhibition by uracil on RNase-IA activity was found to be competitive as determined by Lineweaver-Burk's method.

At a higher concentration of the inhibitor to the substrate (4:1 molar ratio), the bases which revealed strong inhibition was commonly found to possess an enolizable carbonyl group in the pyrimidinone ring, thus it was postulated that an enolizable -CO-NH- group might be the necessary group in these pyrimidinones for its inhibitory action. At this concentration, cytosine was again an exception although it possesses a structure similar to the base-residue of the substrate 2′:3′-cyclic cytidylic acid, because it did not show any affinity to the enzyme. A clarification of this phenomenon awaited further studies. The result was the same for this base when 2′:3′-cyclic uridylate was used as a substrate.

Decreased inhibitory activity by uracil was observed by substitution at its 5-position by an amino group, and increased inhibition was noted when it was substituted by a nitro group. This indicates that the increased dissociation of the enol hydroxyl groups on the pyrimidine ring results in a higher inhibitory property of this series of compounds. Furthermore, the enhanced inhibitory nature of uracil caused by the aminomethyl and dimethylaminomethyl substitution at the 5-position is assumed as the result of the interaction of these basic groups with some of the acidic centers in the active site of the enzyme molecule.

Among the several N_3-ribonucleoside of 2(3H)-pyrimidinones tested, those which had also the structure corresponding to the esters of 3′-pyrimidine nucleotides (known to be a very active substrate for the enzyme) inhibited the enzymatic hydrolysis of 2′:3′-cyclic cytidylic acid. The typical ribofuranosides including uridine, cytidine and 5-bromouridine, were shown to inhibit the enzymatic reaction competitively.

As for the structural effect of β-glycosyl group of nucleosides on inhibitory activity, pentafuranosyl group was found essential. Thus thymine ribo-, 2′-deoxyribo- and xylofuranoside showed high inhibitory activity. The slight decrease in the activity of 2′-deoxyribofuranoside as compared with the corresponding ribofuranoside indicates that the presence of 2′-OH group is responsible in some extent to the increase in affinity of the nucleoside to the enzyme. Furthermore, the epimerization of 3′-OH group of ribothymidine greatly enhances the inhibitory property of the mother ribofuranoside.

The substitution of methyl, bromo or morpholino group at the 5-position of basic residue did not influence the inhibitory nature of uridine. This fact reveals the relative insignificance of these substituents at the 5-position for the affinity of uridine to the enzyme.

The inhibitory property of these ribofuranosides was generally greater than those of the corresponding bases. It is interesting to note that the substitution of a ribosyl or 2′-deoxyribosyl group at the N_3-position of cytosine induced an inhibitory property to the parent base. However, the substitution of a glucosyl group at the N_3-position of thymine markedly diminished the inhibitory activity of the parent base.

The substitution of a phosphoryl group containing a secondary dissociation at one of the hydroxyl groups of ribonucleoside was found to enhance its inhibitory property on cyclic phosphodiesterase activity. And such effect was generally observed for several ribonucleosides irrespective of their base residues, thus adenylic, inosinic as well as guanylic acids exhibited the inhibitory activity although the parent nucleosides were inactive.

In the case of the pyrimidine ribonucleosides, the substitution of the phosphoryl group markedly enhanced the inhibitory property of the parent nucleosides, and this increase in the inhibitory activity was attributed to the increase in the affinity of these phosphates to the active site of the enzyme. Thus the Ki values of uridine 2′(+3′)- and 5′-phosphate were in the order of 10^{-3} comparing with that of 10^{-2} for the parent nucleosides.

Moreover, the observed inhibition indices

reveal that the substitution of the phosphoryl group at 2′(+3′)-hydroxyl group of the pyrimidine ribofuranosides (uridine, cytidine and 5-bromouridine) rather than at their 5′-hydroxyl position seems more effective in increasing their inhibitory property. Therefore, the 2′(+3′)-phosphates of these pyrimidine ribofuranosides were indicated to have a greater affinity to the active site of the enzyme than their corresponding 5′-phosphates. Such a selective effect in the increase of inhibitory activity by the changes in the position of phosphoryl substitution was not generally found for purine nucleotides.

The effect of the type of sugar moiety for the inhibition property was also observed. This was demonstrated in the alteration of the 5′-phosphoryl ribofuranosyl part of ribothymidine 5′-phosphate with 6′-phosphoryl glucopyranosyl residue which resulted in a greatly reduced inhibitory activity.

From the observed effect of the phosphoryl substitution on the inhibitory property of nucleoside, the phosphoryl group is assumed to have a somewhat nonspecific affinity to the active site of the enzyme. This assumption is supported by the observation of the inhibitory action by inorganic phosphate as well as by several organic phosphates other than nucleotides on RNase-IA.

The inorganic phosphate manifested a competitive type of inhibition on RNase-IA. The magnitude of this activity represented by its inhibition index was comparable to that of uracil and its affinity to the enzyme represented by the K*i* value was comparable to that of uridine 2′(+3′)-phosphate. The alteration of phenyl group of phenylphosphate by adenosine and uridine residues decreased, in that order, the K*i* value of the parent compound. Thus, the greatest decrease was observed in the substitution of pyrimidine pentafuranonucleoside. This type of substitution indicates the greatest specific affinity to the active site of the enzyme.

Not one of the phosphodiesters tested, showed any inhibitory activity. The results indicates that the phosphoryl group could play a role in competitive inhibition with the substrate only when it contains a secondary dissociation and that the increase in the number of the phosphoryl dissociation in the molecule of the inhibitor seems to increase the affinity of the inhibitor to the enzyme, thus adenosine 5′-triphosphate gave a smaller K*i* value than that for adenosine 5′-monophosphate.

Our findings, therefore, show that the nucleotides which exhibited a competitive type of inhibition on cyclic phosphodiesterase activity of ribonuclease are constituted of at least three partial structural moieties, each of which indicates specificity in different degrees in its affinity to the active site of the enzyme.

The low inhibition index of methyl-(α+β)-D-ribofuranoside 2(+3)-phosphate, however, reveals that such a partial structure as the combination of sugar and phosphoryl group, *i. e.* phosphoryl sugar residue, will not have a high inhibition index unless a suitable basic residue is substituted at C_1 position of the aldose moiety. Thus, the most important part of the structure of nucleotide for the inhibition of RNase-IA is the base residue attached at C_1′-position of the nucleotide.

Recently, Witzel (*9*) proposed a mechanism describing the transphosphorylase and cyclic phosphodiesterase activities of ribonuclease. He postulated that an intramolecular hydrogen bonding between the carbonyl group at C_2 position of pyrimidinyl ring and the hydroxyl group at C_2′-position of ribose moiety occurred during the reaction, that this bond formation was facilitated by a mesomeric stabilization of the atom grouping in the pyrimidine ring, and that the donation of a proton from the enzyme to phosphate-anion of the substrate was necessary to cause a subsequent concerted reaction.

Dekker (*10*), in his review, suggested that the increase in the basicity of the C_2-carbonyl position of the substrate might be effected by a withdrawal of proton at the N_1 position of uracil or at the C_6-substituted aminogroup of cytosine by an essential basic group in the active center of the RNase molecule.

The present study which screened various

compounds as possible inhibitors for the cyclic phosphodiesterase activity of RNase-IA indicates that the inhibition by a nucleotide depends on its affinity to the active site of the enzyme molecule, that is to say, it depends on the ability of the inhibitor to replace the substrate at the active site of the enzyme. And such property of the inhibitor largely depends on the structure of the base, the sugar residue and the position of the phosphoryl substitution in the sugar moiety of the nucleotide. Also, it is indicated that the active site of the enzyme molecule should have at least three regions comprising the functional groups one of which would respond to one of the three partial structures of the inhibitory nucleotides or that of the substrates.

SUMMARY

This study was undertaken to understand better the mechanism underlining the inhibitory action of nucleotides on ribonuclease-IA activities. A number of 2(3H)-pyrimidinones, purine- and pyrimidine-nucleosides as well as corresponding nucleotides were tested as to their possible inhibition of RNase-IA activity using 2′ : 3′-cyclic cytidylate or uridylate as a substrate.

The representative compounds of each group showed a competitive type of inhibition to the enzymatic reaction. The 2(3H)-pyrimidinones, possessing a structure similar to the basic residue of the substrate nucleotides, exhibited a strong inhibitory activity. This was largely increased by substitution of pentafuranosyl residue at the N_3-position of these pyrimidinones. The pentafuranosyl structure was the most suitable glycosyl residue in the nucleoside for the inhibition of RNase-IA, however no inhibitory activity was observed for all of the purine ribofuranosyl nucleosides tested.

The phosphoryl group substitution at the sugar residue of nucleosides was found significant in the enhancement of the inhibitory function of the parent compound, however, this group did not appear to play an important role in the specific affinity of the parent nucleoside to the enzyme. The location of the phosphoryl group in the sugar residue, however, appeared important for enhancement of the inhibitory activity, and the greatest affinity of the nucleotide to the enzyme was realized when the phosphoryl group was attached at the 2′(or 3′) hydroxyl group of the pentafuranosyl group.

These observations reveal that in the inhibition of cyclic phosphodiesterase activity of RNase-IA by a nucleotide, the structure of each of its three partial residue (the basic group, the sugar residue and the position of phosphoryl substitution) is an important factor in its affinity to an active site of the enzyme molecule. The basic group seems to have the greatest specificity of the three, while the phosphoryl group is somewhat nonspecific in its affinity to the enzyme.

The authors are indebted to Prof. T. Yamada and D. Mizuno of this Faculty and Prof. Y. Mizuno of the University of Hokkaido for their gifts of test compounds.

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Effects of Cyanamide on Alcohol Dehydrogenase and Aldehyde Dehydrogenase

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Cyanamide (H_2NCN) is known as a remedy for chronic alcoholism.

One of the authors observed clinically the "cyanamide-alcohol reaction", that is, the patients who had been administered with cyanamide showed characteristic syndrom, as soon as ethanol was ingested: Nausea and vomiting, an abrupt drop in blood pressure, marked palpitations, and quite regularly a feeling of impending death (*1*).

The authors reinvestigated the effect of the substance on alcohol dehydrogenase and aldehyde dehydrogenase action, though Morimura (*2*) already reported a manometric research that cyanamide inhibited strongly alcohol dehydrogenase, but not aldehyde dehydrogenase.

MATERIALS AND METHODS

Alcohol dehydrogenase was purified from horse liver by Bonnichsen's method (*3*).

The liver extract was heated at 52°C and kept for 15 minutes. The fraction which precipitated between 0.55 and 0.80 saturation of ammonium sulfate was collected, and hemoglobin was removed by the method of Tsuchihashi (*4*). The preparation was then subjected to ethanol fractionation.

Aldehyde dehydrogenase was prepared from beef liver by Racker's method (*5*). Acetone-dried powder of the liver was extracted with ethylenediamine tetraacetate solution. The extract was fractionated with ethanol. After adsorption of the enzyme with RNA, RNA was removed with protamine sulfate. After centrifugation, the supernatant was used as the enzyme preparation.

The activities of both enzymes were measured spectrophotometrically at 340 mμ by estimating the amount of DPN* reduced in every one minute in the presence of the substrates.

The alcohol dehydrogenase reaction mixture contained 0.2 ml. of the enzyme solution, 1×10^{-3} to $5\times10^{-3}\,M$ ethanol, 0.48×10^{-4} to $3.7\times10^{-4}\,M$ DPN in their final concentration in 4.0 ml. of 0.1 *M* glycine-sodium hydroxide buffer, pH 9.6.

The aldehyde dehydrogenase reaction mixture contained 0.5 ml. of the enzyme solution, 1.9×10^{-5} to $7.6\times10^{-5}\,M$ acetaldehyde, 0.6×10^{-4} to $2.5\times10^{-4}\,M$ DPN in their final concentration in 3.0 ml. of 0.1 *M* sodium pyrophosphate buffer, pH 9.3.

RESULTS AND DISCUSSION

The reaction mixtures were incubated at 15°C in quartz glass cells of Beckman spectrophotometer.

In the experiments with alcohol dehydrogenase, the dissociation constant of the substrate from the complex with the enzyme (Ks) was calculated to be $6.4\times10^{-4}\,M$ (Fig. 1) and the dissociation constant of the coenzyme from the complex with apo-enzyme (K_{DPN}) was $2.2\times10^{-4}\,M$ (Fig. 2). It was found that cyanamide did not affect the activity of alcohol dehydrogenase within the range of its concentration from 1.2×10^{-7} to $1.2\times10^{-2}\,M$.

In an experiment with aldehyde dehydrogenase, the final concentration of cynamide in the reaction mixture was $1.6\times10^{-3}\,M$. Ks was calculated to be $10.2\times10^{-6}\,M$ (Fig. 3) and K_{DPN} was $5.9\times10^{-5}\,M$ (Fig. 4). Cyanamide inhibited aldehyde dehydrogenase noncompetitively to the substrate (Fig. 3) as well as to DPN (Fig. 4).

The possibility of the liberation of CN^- from cyanamide *in vivo* was tested by observing the changes in absorption spectra of oxyhemoglobin and met-hemoglobin. The results showed that the cyanamide did not affect

* Abbreviation: DPN, diphosphopyridine nucleotide.

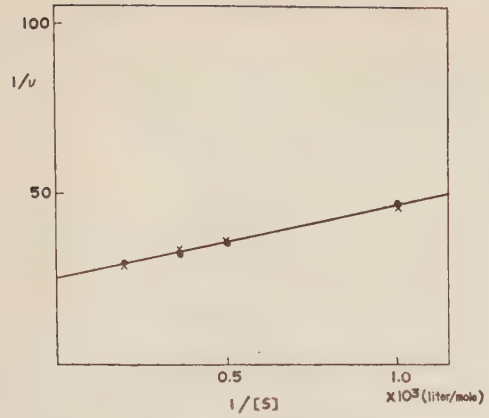

Fig. 1. Lineweaver-Burk plots of alcohol dehydrogenase activity against the concentration of substrate.

—●—, Reaction mixture was composed of 0.2 ml. of alcohol dehydrogenase solution, 0.2 ml. of ethanol solution, 0.1 ml. of $1.9\times10^{-4}M$ DPN solution, 3.3 ml. of glycine-sodium hydro xide buffer, 0.2 ml. of water.

—×—, In stead of water of the above-mentioned system, 0.2 ml. of cyanamide solution was added.

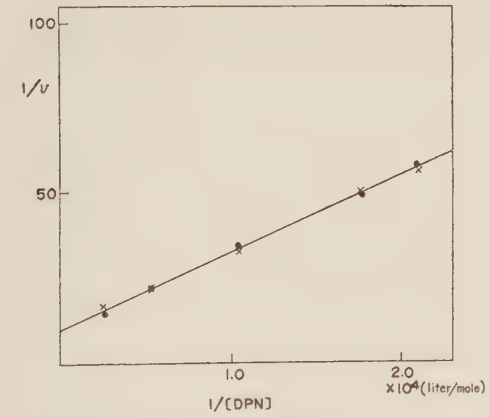

Fig. 2. Lineweaver-Burk plots of alcohol dehydrogenase activity against the concentration of DPN.

—●—, Reaction mixture was composed of 0.2 ml. of alcohol dehydrogenase solution, 0.2 ml. of 1 M ethanol solution, 0.1 ml. of DPN solution, 3.3 ml. of glycine-sodium hydroxide buffer, 0.2 ml. of water.

—×—, In stead of water of the above-mentioned system, 0.2 ml. cyanamide solution was added.

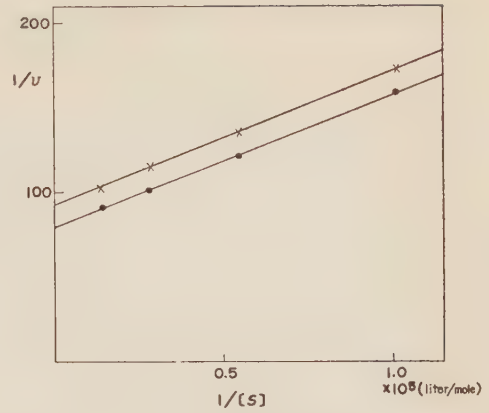

Fig. 3. Lineweaver-Burk plots of aldehyde dehydrogenase activity against the concentration of substrate.

—●—, Reaction mixture was composed of 0.5 ml. of aldehyde dehydrogenase solution, 0.1 ml. of acetaldehyde solution, 0.1 ml. of 2.5×10^{-4} M DPN solution, 0.3 ml. of sodium pyrophosphate buffer, 2.0 ml. of water.

—×—, Reaction mixture was composed of 0.5 ml. of the enzyme solution, 0.1 ml. of acetaldehyde solution, 0.1 ml. of $2.5\times10^{-4}M$ DPN solution, 0.3 ml. of the buffer, 0.2 ml. of $1.6\times10^{-3}M$ cyanamide solution, 1.8 ml. of water.

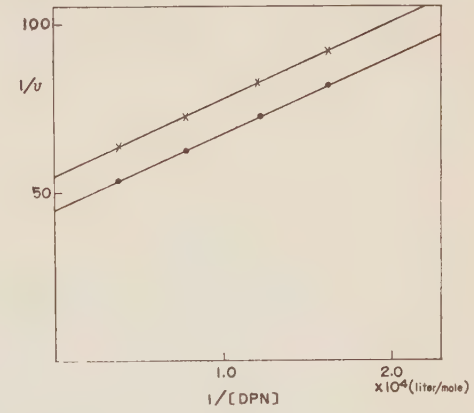

Fig. 4. Lineweaver-Burk plots of aldehyde dehydrogenase activity against the concentration of DPN.

—●—, Reaction mixture was composed of 0.5 ml. of aldehyde dehydrogenase solution, 0.1 ml. of $3.8\times10^{-3}M$ acetaldehyde solution, 0.1 ml. of DPN solution, 0.3 ml. of sodium pyrophosphate buffer, 2.0 ml. of water.

—×—, Reaction mixture was composed of 0.5 ml. of the enzyme solution, 0.1 ml. of 3.8×10^{-3}

M acetaldehyde solution, 0.1 ml. of DPN solution, 0.3 ml. of the buffer, 0.2 ml. of 1.6×10^{-3} *M* cyanamide solution, 1.8 ml. of water.

the spectra, suggesting that the liberation of CN^- from cyanamide could be excluded.

In these experiments, the authors could not find that cyanamide inhibited alcohol dehydrogenase and did not affect aldehyde dehydrogenase. The authors consider that the activities of alcohol dehydrogenase and aldehyde dehydrogenase can be specifically measured by spectrophotometric method.

In the previous medical researches on cyanamide, most of them used cyanamide in powder or solid state. But this is apt to be rapidly polymerized (dicyan diamine) and loses its pharmacological action. In authors' experiments, cyanamide which had been stored as soluble state in water was used. This is quite stable.

The fact that cyanamide inhibits aldehyde dehydrogenase seems to be an important factor of the mechanism of the cyanamide-alcohol reaction. Assuming that this inhibitory action is taken place *in vivo*, it may result in the elevation of the concentration of acetaldehyde in living body. This possibility corresponds with the autonomic nerve symptoms of cyanamide-alcohol reaction. Therefore, cyanamide may be considered to be one of the remedies of conditioned-reflex therapy such as tetraethylthiuram disulfide.

SUMMARY

1. The effects of cyanamide on alcohol dehydrogenase and aldehyde dehydrogenase were studied.
2. Cyanamide did not affect the activity of alcohol dehydrogenase.
3. Cyanamide inhibited aldehyde dehydrogenase noncompetitively to acetaldehyde as well as to the coenzyme.

The authors wish to thank Dr. K. Yagi for his guidance and valuable criticisms.

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Further Studies on Microsomal Lactonase

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It was reported by Winkelman and Lehninger (*1*) that uronolactonase was found in microsomes but not in the soluble fraction of rat liver, and that it hydrolyzed D-glucurono-γ-lactone but was inactive against a wide variety of aldonolactones. This enzyme was inhibited by ethylenediamine tetraacetate, but the inhibition was relieved by Mn^{++}, Mg^{++}, Zn^{++}, Co^{++} and Fe^{++}. Yamada *et al.* (*2, 3*) reported independently the presence of lactonase II in microsomes of rat liver which hydrolyzed D-glucurono-γ-lactone but not aldonolactones. This enzyme differed from soluble lactonase, named lactonase I, in intracellular distribution, substrate specificity and alkali lability.

In the previous paper of the present authors (*4*), it was shown that drugs which stimulated the excretion of L-ascorbic acid, such as Chloretone, antipyrine and barbital, increased the activity of lactonase II. However, in the conversion of D-glucuronic acid to L-ascorbic acid, the physiological significance of lactonase II has not yet been clarified.

In the present paper the properties of lactonase II was studied further and its physiological significance was discussed.

EXPERIMENTAL

Chemicals—D-Ribono-γ-lactone, D-gulono-γ-lactone, L-gulono-γ-lactone and D-galactono-γ-lactone were purchased from Nutritional Biochemicals Corporation. D-Mannono-γ-lactone was generously donated by Dr. H. S. Isbell of the National Bureau of Standards, U. S. A. D-Glucurono-γ-lactone was obtained from the Chugai Pharmaceutical Company. D-Mannurono-γ-lactone was obtained from the Takeda Chemical Industries. D-Idurono-γ-lactone was donated by Mr. S. Takanashi of the Reseach Laboratory of Chugai Pharmaceutical Company.

Preparation of the Cell Components—Fractions of cell component, namely mitochondria, heavy microsomes, light microsomes, and the soluble fraction, were prepared by the method of Hogeboom (*5*) with some modifications. In the present work, the two layer method of 0.25 *M* and 0.35 *M* sucrose was applied for the isolation of the mitochondrial fraction, although Hogeboom used this method to isolate a nuclear fraction.

Isolation of heavy microsomes: The combined supernatants obtained from 1 g. of liver in the isolation procedure of mitochondria were made up to a volume of 35 ml. with 0.25 *M* sucrose, and the pH of the solution was adjusted to 6.7 with 0.1 *M* tris (hydroxymethyl) aminomethane buffer. The firmly packed pellet was obtained by the centrifugation for 60 minutes at $23,000 \times g$, and it was resuspended in 1 ml. of 0.25 *M* sucrose per 1 g. of wet liver.

Isolation of light microsomes: The supernatant obtained in the isolation procedure of heavy microsomes was centrifuged for 60 minutes at $54,000 \times g$. The firmly packed pellet was obtained and it was resuspended in 1 ml. of 0.25 *M* sucrose per 1 g. of wet liver.

Assay Methods

Manometric Method—The enzyme was assayed manometrically by the measurement of carbon dioxide evolution in a bicarbonate-carbon dioxide buffered solution. Conditions were as follows: Reaction mixture contained 0.4 ml. of 0.1 *M* sodium bicarbonate, 0.1 ml. of 0.01 *M* magnesium sulfate, 0.3 ml. of 0.001 *M* glutathione and the microsomes obtained from 1 g. of wet liver, and 0.3 ml. of 0.1 *M* substrate was added from the side arm. The total volume was made to 3 ml. Gas phase, 93 per cent of N_2 and 7 per cent of CO_2; temperature, 37°C; pH 7.2. The substrate was tipped in after preincubation for 10 minutes.

Colorimetric Method—In case where it was important to know exactly the pH of the reaction mixture, the enzyme activity was measured by the estimation of residual lactone. The estimation was carried out by

an adaptation of the hydroxamic acid procedure of Eisenberg (*6*) as follows: Neutral hydroxamine was prepared each day from 4 parts of 4 *M* hydroxamine hydrochloride and 3.5 parts of 3.5 *M* sodium hydroxide. An equal volume of 0.1 *M* acetate buffer (pH 5.4) was then added. One tenth ml. aliquot of the sample to be analyzed was diluted with water to 1 ml. and it was added to 1 ml. of the buffered hydroxamine reagent in small test tubes. The solution was allowed to stand for 10 minutes to complete the formation of the hydroxamic acid derivative, after which 1 ml. of the reagent containing 24 per cent of concentrated hydrochloric acid, 6 per cent of trichloroacetic acid, and 17 per cent of $FeCl_3 \cdot 6\,H_2O$ were added. The tubes were centrifuged for 5 minutes. After 60 minute standing for the development of color, the absorbancy at 540 mμ of the clear supernatant was measured with Beckman spectrophotometer.

In order to assay the lactonizing activity, hydroxylamine hydrochloride was used as a trapping agent and hydroxamate formed from the lactone was measured colorimetrically as follows: Hydroxylamine hydrochloride solution was freshly prepared every day. Its pH was adjusted to that of the reaction mixture by the addition of 4 *M* sodium hydroxide, and the concentration of the solution was brought to 2 *M* by the addition of water. After the reaction proceeded under the conditions described in the legend of Fig. 5, 2 ml. aliquot of the reaction mixture was mixed with 1 ml. of the abovementioned reagent containing $FeCl_3$. The above method was followed to estimate the formed lactone.

Unit of Enzyme Activity—Unit of enzyme activity was defined as the activity that produced 1 micromole of carbon dioxide during 10 minutes in the manometric determination.

RESULT

Intracellular Distribution of Lactonase II—The distribution of the lactonase activity on D-glucurono-γ-lactone was examined with mitochondria and heavy and light microsomes. It was found that the activity was high in heavy microsomes as shown in Table I.

TABLE I

Intracellular Distribution of Lactonase II

Cell fraction	Mitochondria	Heavy microsomes	Light microsomes
Activity	0	9.9	0.4

The figures show the unit of lactonase activity on D-glucurono-γ-lactone per 1 g. of wet liver.

Properties of Lactonase II

Effect of pH—The optimum pH for the D-glucuronolactone hydrolysis was found to be about 7.2 with phosphate buffer at 37°C. At pH 6.3 the enzymatic hydrolysis of glucuronolactone disappeared completely. At the pH range over 7.6 the enzymatic hydrolysis could not be estimated exactly, since the nonenzymatic hydrolysis appeared markedly.

Stability—Heating of the microsomes at 65°C for 3 minutes destroyed completely the activity against D-glucurono-γ-lactone, while it reduced only about 20 per cent of the activity aginst D-gulonolactone and D-galactonolactone (Fig. 1).

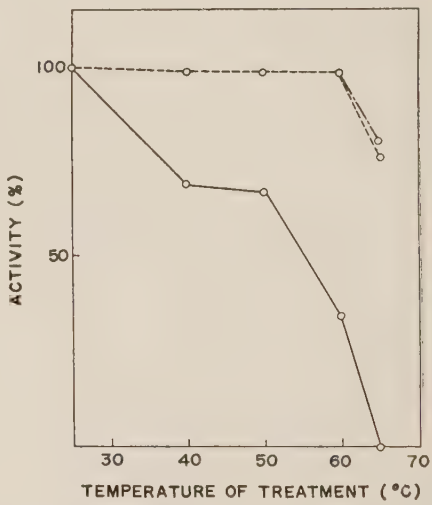

FIG. 1. Effect of heat treatment. Microsomes were heated for 3 minutes at the specified temperature, and then the lactonase activity was determined on D-glucuronolactone, D-gulonolactone and D-galactonolactone as substrate.

——○—— D-glucuronolactone
—·—○—·— D-gulonolactone
----○---- D-galactonolactone

Alkali treatment of the microsomes in 0.1 *M* sodium bicarbonate solution at pH 9.0 at 37°C for 3 hours destroyed completly the activity against D-glucurono-γ-lactone, while it did not affect the activity on D-galactono-γ-lactone (Fig. 2).

Aging of the microsomes at 5°C almost

destroyed the activity against D-glucuronolactone, while it reduced about 50 per cent of the activity against D-galactonolactone (Fig. 3).

Freezing and thawing did not affect the activity against D-glucuronolactone. It could be repeated five times without any change in the activity.

Activation and Inhibition—The addition of $10^{-3}\,M$ of Mg^{++} ions to the reaction mixture caused a 45% stimulation of the lactonase activity against D-glucuronolactone. The addition of $10^{-3}\sim10^{-4}\,M$ of Mn^{++}, Co^{++} and Cu^{++} ions caused the inhibition of the activity (Fig. 4). The inhibition was also caused by the addition of *p*-chloromercuribenzoate. The reversal of this inhibition occurred if glutathione was added, and this effect of glutathione was dependent on the presence of Mg^{++} ion.

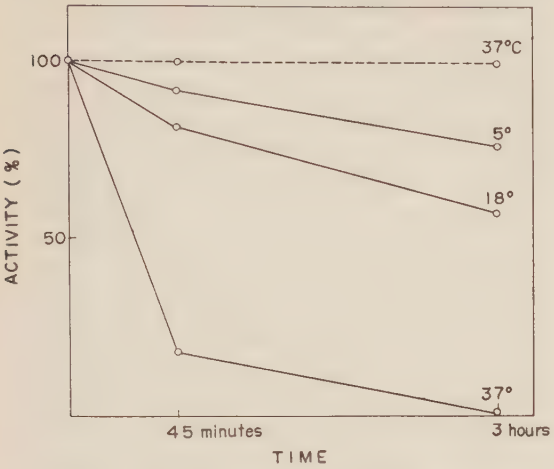

FIG. 2. Effect of alkali treatment. Microsomes were preincubated in 0.1 *M* $NaHCO_3$ at pH 9.0 at various temperatures for 45 minutes or 3 hours, and the lactonase activity was measured on D-glucuronolactone and D-galactonolactone as substrate.

——○—— D-glucuronolactone
----○---- D-galactonolactone

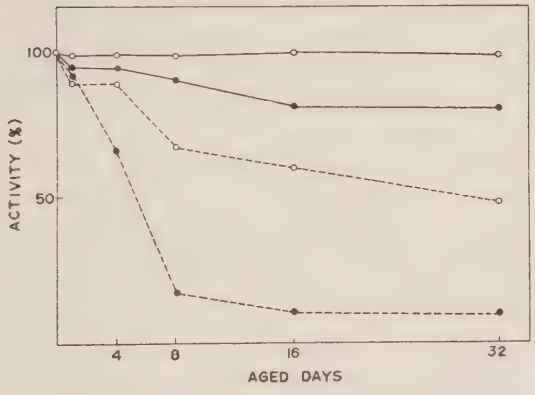

FIG. 3. Lactonase activity of aged microsomes. The aging of the microsomes was carried out at 5°C or −20°C for 32 days and the lactonase activity was determined on D-glucuronolactone and D-galactonolactone as substrates. In this assay 0.1 *M* $MgSO_4$ was used instead of 0.01 *M* $MgSO_4$.

----●---- D-glucuronolactone + microsomes aged at 5°C
——●—— D-glucuronolactone + microsomes aged at −20°C
----○---- D-galactonolactone + microsomes aged at 5°C
——○—— D-galactonolactone + microsomes aged at −20°C

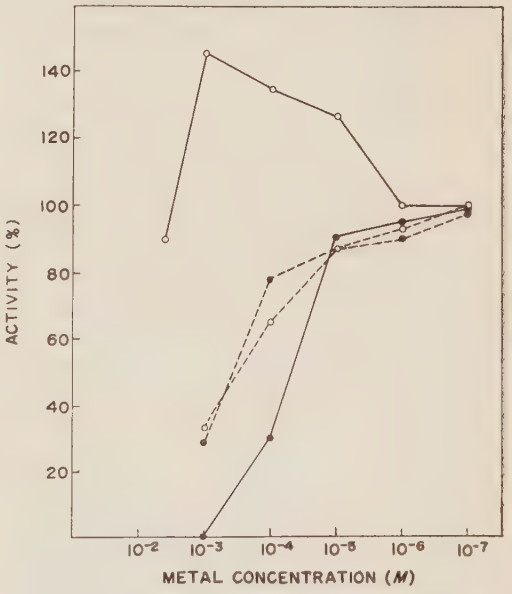

FIG. 4. Effect of metal on lactonase II activity. The lactonase activity was determined on D-glucuronolactone under the addition of the metal ions in various concentrations.

——○—— Mg^{++}
----○---- Mn^{++}
——●—— Cu^{++}
----●---- Co^{++}

Back Reaction—The back reaction was studied at pH 6.4 with tris (hydroxymethyl) aminomethane-maleate buffer by the addition of 0.2 *M* hydroxylamine (pH 6.4). The observed time course of the lactone formation

is shown in Fig. 5. A little more lactone was formed when microsomes were added to the reaction mixture than in their absence.

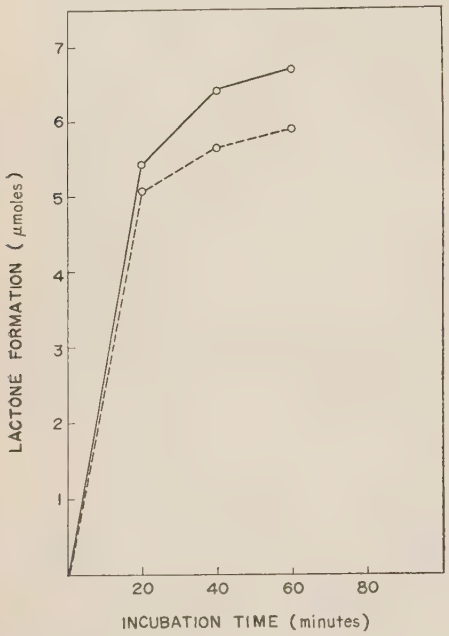

FIG. 5. Assay of lactonizing activity. Each vessel contained: 0.05 *M* tris (hydroxymethyl) aminomethane-maleate buffer (pH 6.4), 4 ml.; 0.1 *M* $MgSO_4$, 0.2 ml.; 2 *M* hydroxylamine (adjusted to pH 6.4) 0.6 ml.; 0.01 *M* glutathione, 0.1 ml.; microsomes obtained from 1 g. wet liver; sodium glucuronate 120 μmole; total volume, 6.0 ml. Incubation; 37°C.
——○—— enzymatic
- - -○- - - non-enzymatic

Substrate Affinity Constant—The Michaelis constant, calculated by the method of Lineweaver and Burk (*7*), was found to be 1.78×10^{-2} mole per liter for D-glucuronolactone. This value is higher than that of uronolactonase reported by Winkelman and Lehninger (*1*).

Substrate Specificity—The lactonase activity of heavy microsomes on D-glucurono-γ-lactone was considerably higher than that on other lactones, such as D- and L-gulono-, D-galactono-, D-mannono-, D-ribono-, D-mannurono- and L-idurono-lactone. When this microsomal fraction was resuspended in 0.25 *M* sucrose and subjected to centrifugation twice, the reduction proportion of the activity on D-glucuronolactone was far smaller than that on D- and L-gulonolactone and D-galactonolactone. The activity on D-glucurono-γ-lactone of untreated microsomes markedly differed from that of microsomes which was heated at 65°C for 3 minutes, while both microsomes showed no difference in the activity on other lactones. The specificity of this microsomal preparation resembled that of lactonase I reported by Yamada (*3*), except for the high activity against D-glucurono-γ-lactone. Microsomes of human and monkey (*Macaca cynomolga* Linne) liver in which the activity of lactonase I was weak did not act on other substrates than D-glucuronolactone. These findings show the possibility that the activity against the other substrates than D-glucurono-γ-lactone came from the contamination of lactonase I in the microsomal preparation. Lactonase II seems to act only on D-glucurono-γ-lactone and seems not to act on D- and L-gulono-, D-mannono- and D-mannurono-lactone as already suggested by Yamada *et al.* (*2, 3*). Furthermore it was found that the microsomal lactonase seemed not to act on L-idurono-γ-lactone and D-ribono-γ-lactone.

DISCUSSION

Winkelman and Lehninger (*1*) reported that aldonolactonase differed from uronolactonase in tissue distribution, intracellular location and substrate specificity. Yamada *et al.* (*2, 3*) differentiated lactonase I and II on the basis of their differences in intracellular distribution, substrate specificity and alkali lability. In the present experiment the difference of lactonase I and II could be also observed in the lability against heat treatment and aging of lactonase II and their difference in the effect of metal on the activity.

The formation of D-glucuronic acid from uridine diphosphate glucuronic acid was observed with the particulate preparation of rat kidney or rat skin homogenate (*8, 9*). In the present experiment it was found that lactonase II catalyzed the conversion of D-

glucuronic acid to glucuronolactone. It was reported that the microsomes of rat liver, in the presence of cyanide under anaerobic condition, was able to convert D-glucuronolactone to L-gulonolactone which in turn was converted under the aerobic condition to L-ascorbic acid promptly (*10–12*). To supply glucuronolactone in the above reaction, lactonase II located in microsomes would be favourable to lactonization of glucuronic acid considering the intracellular location. The previous report (*4*) showed that the drugs which stimulated the excretion of L-ascorbic acid, such as sodium 5,5-diethylbarbiturate, antipyrine and Chloretone, increased the activity of lactonase II, and the excretion of L-ascorbic acid and the activity of lactonase II reached maximum on 7~8 days after the beginning of sodium 5,5-diethylbarbiturate administration. These results suggest the possibility that lactonase II plays some role in biosynthesis of L-ascorbic acid.

On the other hand, the experiment of the reconstructed system showed that the system consisted of TPN L-hexonate dehydrogenase and microsomal enzyme could not form L-ascorbic acid from glucuronic acid without the addition of lactonase I (*13*). Therefore further investigation seems to be required before the physiological significance of lactonization by lactonase II is clarified.

The further studies on this enzyme through the solubilization of microsomes is now under investigation.

SUMMARY

1. Lactonase II located in the microsomes was studied. Its optimum pH was at about 7.2, and it was proved to be a SH enzyme.

2. Lactonase II was shown to be very labile against heat and alkali treatment and aging.

3. Lactonase II seemed to act only on D-glucurono-γ-lactone, but not on D-mannurono-γ-lactone and L-idurono-γ-lactone. It seemed not to act on aldonolactone.

4. The significance of this enzyme in the L-ascorbic acid biosynthesis was discussed.

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Dimethylalloxan as a Reagent for Paper Chromatography of Amino Acids

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It is known that on oxidation of purines with nitric acid, alloxan derivatives are formed. When ammonium hydroxide is added to the reaction mixture, a violet-red color is developed and this reaction is known as the "murexide reaction" for a qualitative test of purine derivatives (*1*). It is also well known that a colored murexide and the corresponding aldehyde are formed on the reaction of an amino acid with alloxan (*2*). The use of alloxan for the detection of amino acids, with the exception of proline and hydroxyproline, in paper chromatography has been reported by Saifer and Oreskes (*3*). The present authors have found that dimethylalloxan gave a relatively stable pinkish red color with amino acids including proline on paper and that the reagent was superior than the alloxan reagent in sensitivity and was as useful as ninhydrin for identifying amino acids by paper chromatography or paper electrophoresis. Several properties of the colored murexide and a simple preparation method of dimethylalloxan from caffeine are described in the present communication.

EXPERIMENTALS

Preparation of Dimethylalloxan—A mixture of 100 g. of caffeine and 50 ml. of acetic acid was heated for a while in a round bottom flask of 500 ml. to dissolve a part of the caffeine. When a mixture of 80 ml. of fuming nitric acid (d, 1.50) and 80 ml. of acetic acid was added in the flask dropwise, vigorous reaction immediately started with heat evolving. Acetic acid vapor was collected with a condenser and the reaction was allowed to proceed by controlling the addition speed of the acid mixture and by cooling the reaction mixture. During the reaction special precaution was taken to prevent an explosion owing to the vigorous reaction. After the reaction was complete, an orange yellow liquid in the flask solidified. Dimethylalloxan crystallized was filtered off and washed successively with acetic acid and benzene. On recrystallization from water, about 70 g. of white hydrated crystals were obtained.

Anal: Calcd. for $C_6H_6O_4N_2 \cdot 2H_2O$: N, 16.46; H_2O, 17.48.

Found: N, 16.62; H_2O, 17.57.

When the material was dried over phosphorous pentoxide at 100°C *in vacuo*, hydrated water was lost. M.p. 270°C, uncorr. (reported m. p. 270~272°C (*4*)).

Anal: Calcd. for $C_6H_6O_4N_2$: C, 42.36; H, 3.56.

Found: C, 43.04; H, 3.56.

Dimethylalloxan Reagent for Paper Chromatography—The reagent solution was 0.5% dimethylalloxan in absolute ethanol or acetone. It was sprayed on a dry paper chromatogram and the paper was heated at 100°C for 5 minutes. The maximal red color appeared within 2 to 3 minutes, but heating more than 120°C was avoided because the background become slightly colored. The reagent solution was not stable at room temperature but could be stored in a refrigerator for about a week without appreciable decrease of its usefulness. However the fresh solution was prepared every day.

RESULTS AND DISCUSSION

Several authentic amino acids were chromatographed one-dimensionally with *n*-butanol-acetic acid-water (4 : 1 : 1, by volume) on paper and the chromatogram was made by spraying the dimethylalloxan reagent as described in the experimental part. Color shades of the spots on the paper chromatogram were slightly different from one amino acid to another. Some of them are listed in Table I. Table I also shows the minimum quantities of some amino acids necessary for

TABLE I

Minimum Quantities of Some Amino Acids Necessary for the Detection with the Dimethylalloxan Reagent

Amino acid	0.5% Dimethylalloxan in ethanol[1]			0.1% Ninhydrin in *n*-butanol[2]	
	μg.	(μmole)		μg.	
Aspartic acid	0.7	(0.005)	red	0.4	blue
Asparagine	0.7	(0.005)	red	1	brown yellow
Cystine	1.2	(0.005)	brown red		
Histidine	0.8	(0.005)	purple red	25	brown purple
Hydroxyproline	no reaction[3]			1	orange yellow
Leucine	0.7	(0.005)	red	0.5	purple
Lysine	0.8	(0.005)	purple red	3	purple
Proline	5.8	(0.05)	grey red	1	yellow
Serine	0.5	(0.005)	red	0.3	red purple
Tyrosine	0.9	(0.005)	brown red	3	brown purple

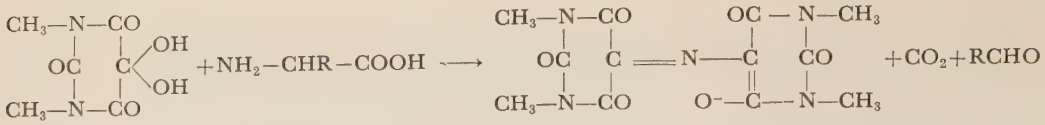
1) One-dimensional paper chromatogram developed with *n*-butanol-acetic acid-water (4 : 1 : 1) was sprayed with the reagent and heated at 100°C for 5 minutes.

2) Data were transfered from the paper of Pratt and Auclair (*5*). Two-dimensional paper chromatogram developed with phenol and collidine-lutidine (1 : 1) was sprayed with the reagent and heated at 100°C for 5 minutes.

3) Up to 0.5 μmole hydroxyproline did not give color.

the detection on a paper chromatogram. The quantities are compared with those detected by ninhydrin reported by Pratt and Auclair (*5*). In the latter case they used two-dimensional chromatogram instead of one-dimensional used in the present experiments. With the minimum quantities of amino acids necessary for the dimethylalloxan reaction, the ninhydrin reagent also gave detectable purple colors on a chromatogram. It is obvious from the results described above that the sensitivity of dimethylalloxan is almost the same as or slightly less than that of ninhydrin. The exception is hydroxyproline which does not give color with the former reagent up to 0.5 μmoles. However the colored spots of the other amino acids with dimethylalloxan are more stable than those with ninhydrin. For the comparison of the stabilities of the colored materials formed with dimethylalloxan and with ninhydrin (0.2 per cent in *n*-butanol saturated with water), two paper chromatograms were made by using 0.1 μmole of each of several amino acids. The one was colored with the former reagent and the other with the latter reagent, and both chromotograms were kept under the same conditions at room temperature. The colored spots with ninhydrin faded rather rapidly and some times disappeared after a week or two. Contrary to ninhydrin, the red color with dimethylalloxan faded quite slowly as it progressed from brownish orange to yellow, but after two to three months the colored spots were still clearly recognizable without any treatment. ω-Amino acids such as β-alanine, typical peptides, proteins, and amino acid hydrazides also reacted with dimethylalloxan and gave red colors on a paper. The sensitivity of the reagent for amino acid hydrazides was far better than that of ninhydrin.

As the structure of dimethylalloxan resembles ninhydrin closely, it seems that the colored compound, "murexide", formed by the reaction of the former reagent with an amino acid has the similar structure with the "Ruhemann's purple" and that the reaction proceeds according to the following scheme:



If the final products of the reaction of common amino acids with dimethylalloxan were the same as is shown in the above scheme, the color shades of the murexides formed from various amino acids to be the same with each other. However, the color shades of the amino acid spots on a paper chromatogram were different from one amino acid to another as is recorded in Table I. This phenomenon has been observed and recognized widely on the ninhydrin reaction on a paper. Recently Tominaga (*6*) of the author's laboratory found that the ninhydrin reaction with amino acids on a paper accompanied by several side reactions, whose products also colored, besides the main reaction which leads to the Ruhemann's purple, and that the products of the side reactions were different from one amino acid to another. However, when the ninhydrin reaction was performed in solution, no product due to the side reaction was found in the reaction mixture. The above findings of Tominaga will explain the different color shades of the amino acid spots with ninhydrin on a paper. The above explanation for the ninhydrin reaction will be able to extend to the present dimethylalloxan reaction.

During the preparation of the present manuscript, the authors found that Saifer and Oreskes (*3*) had reported on the alloxan reagent for paper chromatography of amino acids. They described almost the same results as the present dimethylalloxan reagent, but they reported that proline did not react with the reagent. Therefore the sensitivity of the alloxan reagent for amino acids, especially for proline, was compared with that of the dimethylalloxan reagent. The colors of the amino acids with alloxan were almost the same as that with dimethylalloxan. The intensities of the colors of amino acid spots on a chromatogram were measured by the use of densitometer. The area under the peak which corresponded to the colored spot obtained by the reaction of serine with alloxan was about a half of that with dimethylalloxan, but that of proline with alloxan was about a quarter of that with dimethylalloxan. Therefore the dimethylalloxan reagent is superior than the alloxan reagent.

In order to measure an absorption spectrum of the colored material formed with an amino acid and dimethylalloxan reagent, the red spot on a paper chromatogram was extracted into a test tube with 5 ml. of various concentrations of aqueous ethanol for 4 hours at room temperature in the dark. The spectrum was recorded by the Cary Automatic Recording Spectrophotometer. As is shown in Fig. 1, an absorption maximum

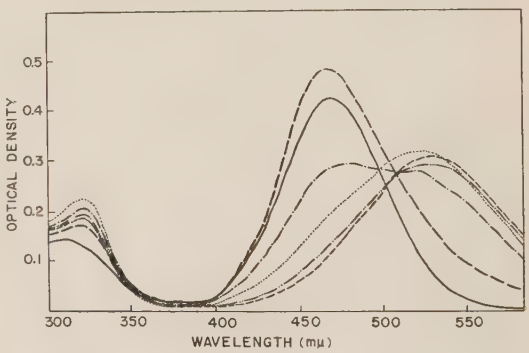


Fig. 1. Absorption spectra of the colored compound produced by the reaction of serine with dimethylalloxan in various cencentrations of aqueous ethanol. 0.5 per cent of dimethylalloxan in absolute ethanol on a paper and the red color was extracted with absolute ethanol (——), 80% (— —), 60% (—·—), 40% (·····), 20% ethanol (—··—) and water (- - - -).

(470 mμ in absolute ethanol) shifts to a longer wave length (530 mμ in water) with the increase of water content in ethanol. Solubility of the murexide increased with the increase of water content in ethanol, and when absolute ethanol was used, complete extraction needed about 24 hours. In Fig. 1, the fact that the optical density of the murexide extracted with

absolute ethanol from the paper is lower than that extracted with 80% ethanol suggests incomplete extraction. This phenomenon is also shown in Fig. 2 which records the stability of the murexide measured at 470 mμ and at 525 mμ in various concentrations of aqueous ethanol. A maximum measured at 470 mμ after extraction for a period of 25 hours seems to indicate complete extraction of the compound. Stability of the murexide rapidly decreased with the increase of water content in ethanol. Therefore if suitable conditions were chosen to react amino acid with dimethylalloxan on a filter paper and if ethanol is used for the extraction of the murexide formed, semi-quantitative amino acid analysis by paper chromatography is possible.

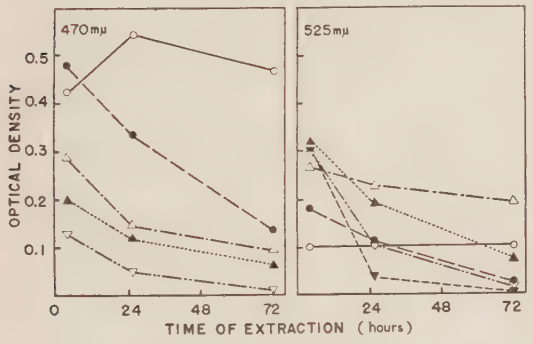


FIG. 2. Stability of the murexide in various concentrations of aqueous ethanol. Absolute ethanol (——), 80% (— —), 60% (—·—), 40% (·····), 20% ethanol (—··—) and water (– – – –).

An attempt was made to estimate amino acids in solution, such as in effluents from column chromatography, by using the dimethylalloxan reagent, as it would replace the ninhydrin reagent which is used for the automatic amino acid analyzer. One sample of an amino acid (for instance serine, 0.4 μmoles per ml.) was treated with ninhydrin (0.5 ml. of the amino acid solution+1 ml. of 2% ninhydrin reagent (*7*), heated at 100°C for 15 minutes and diluted with 5 ml. of 50% aqueous ethanol), and the other with dimethylalloxan (0.5 ml. of the amino acid solution +6 ml. of 0.5% dimethylalloxan in ethanol, heated for 5 minutes at 100°C). A maximum of the optical density of the reaction mixture with dimethylalloxan was only a quarter of that with ninhydrin. The color intensity developed with dimethylalloxan differed with slight changes of heating conditions, and reproducible readings could not be obtained. A slight variation of the composition of the solvent used for the coloring reaction brought about a shift in the absorption maximum of the murexide formed. Although several conditions were tried, no suitable ones to estimate amino acids quantitatively in solution could be found thus far.

SUMMARY

It was shown that 0.5% dimethylalloxan in ethanol was useful as a detecting reagent for paper chromatography of amino acids and peptides. On heating the paper chromatogram which was sprayed with the dimethylalloxan reagent, amino acids appeared as stable pinkish red spots. Sensitivity of the reagent was almost the same or slightly less than that of the ninhydrin reagent for most amino acids occurring in proteins. Stability of the colored material formed on a paper chromatogram was far better than that with ninhydrin. However, the pinkish red color was quite unstable in aqueous solution and no suitable conditions for the quantitative estimation of amino acids in solution could be found.

The present work has been aided in part by Tanabe Essential Amino Acids Research Foundation for which the authors are grateful.

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Enzymatic Hydrolysis of N-Palmitoyl-Amino Acids by *Mycobacterium avium*

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(Received for publication, July 1, 1961)

N-Acyl-amino acid acylase is widely distributed in animal organs and microorganisms, and has been extensively studied by many workers. According to Birnbaum (*1*), acylases in hog kidney fractions, hydrolyzing N-chloroacetyl or N-acetyl derivatives of various amino acids, were classified into acylase I, II, and III with respect to the amino acid moiety in the substrate.

On the hydrolysis of long chain acyl-amino acids, several researches have been reported: Bondi and Frankl (*2*) studied the hydrolysis of lauroyl-glycine and -alanine in liver and kidney, and later Izar (*3*) worked on the hydrolysis of lauroyl-, myristoyl-, palmitoyl-, and stearoyl-compounds of glycine or alanine by extracts of animal organs and confirmed that liver and kidney had an activity to decompose these long chain acyl-amino acids. Neuberg and Mandl (*4*), using the commercial acylases, studied the hydrolysis of lauroyl- and palmitoyl-amino acids. On Mycobacteria, lauroyl-glycine and -phenylalanine were hydrolyzed by *M. phlei* (*5*), and α,ε-di-caproyl-lysine by *M. avium* (*6*). Recently, Fukui and Axelrod (*7*) reported the hydrolysis of N-palmitoyl-L-phenylalanine by a rat liver preparation.

Mycobacteria contain specific lipids which have characteristic biological and pathogenic effects in animal bodies (*8-10*). The present author has been interested in enzymatic reaction of higher fatty acids in this organism, and using N-palmitoyl-amino acids as substrates the hydrolysis by the partially purified preparation from *M. avium* was studied. In this report, the enzymes catalyzing the hydrolysis of N-palmitoyl-amino acids were distinguished from the conventional acylases hydrolyzing lower acyl-amino acids.

EXPERIMENTAL

Materials—N-Acetyl-DL-valine (m.p. 148°C), N-acetyl-L-aspartic acid (m. p. 145°C), and N-chloroacetyl-L-phenylalanine (m.p. 127°C) were synthesised according to Greenstein (*11*). N-Palmitoyl-DL-valine (m.p. 84°C) and N-palmitoyl-L-aspartic acid (m.p. 122°C) were prepared by the acid chloride method, and N-palmitoyl-L-phenylalanine (m.p. 77°C) by the mixed anhydride method. Acetyl-aspartic acid and chloroacetyl-phenylalanine were kindly furnished by Dr. S. Sakakibara of The Institute for Protein Research, Osaka University, and palmitoyl-amino acids were kindly furnished by Drs. A. Tanaka, I. Higashi, and T. Tsubone of the Department of Medical Chemistry, Faculty of Medicine, Kyushu University.

Highly purified Taka-acylase preparation was a gift of Dr. I. Kato of The Department of Chemistry, Faculty of Science, Osaka University.

Assay of Enzymatic Activities—The hydrolytic activities of the enzyme for acyl-amino acids were estimated colorimetrically (570 mμ) by the ninhydrin method of Yemm and Cocking (*12*) from the liberated amino acids.

The standard assay mixture contained 0.1 ml. of 0.05 *M* acyl-L-amino acid adjusted to pH 7.0, 0.5 ml. of 0.2 *M* phosphate buffer, pH 7.0, and water in total volume of 2.0 ml. When the acyl-aspartic acid was used as substrate, the pH of phosphate buffer was 6.1. Usually the reaction was started by adding the enzyme.

The incubation was carried out at 37.5°C. After 40 minutes incubation, the reaction was stopped by adding 5.0 ml. of 0.2 *M* citric acid-0.425 *M* NaOH buffer, pH 5.0, followed by heating in boiling water-bath for two minutes. The coagulated palmitoyl-amino acids and denatured enzyme were removed by folded filter paper, Toyo Filter Paper No. 131, with the aid of a small amount of Hyflo Super Cel, and 1.0 ml. of the filtrate was used for the estimation of

the liberated amino acid.

Protein nitrogen was estimated by the micro-Kjeldahl method.

RESULTS

Purification of the Enzyme Catalyzing the Hydrolysis of Acyl-Amino Acid—Mycobacterium avium (strain Takeo) was grown in glyceroyl-bouillon medium for five days. The cells were washed by water, followed by several volumes of cold acetone (−15°C) for two times. The cells were desiccated *in vacuo* until the solvent was completely evaporated.

This acetone-dried cells were ground by a motor-driven mill with two portions of sea-sand for 60 minutes and subsequently extracted with 10 volumes of 0.02 *M* phosphate buffer, pH 7.0. After standing overnight in a refrigerator, the cells were centrifuged at 35,000×*g* for 20 minutes to remove cellular debris and the supernatant fluid was used as crude extract.

The crude extract was brought to pH 5.3 by careful addition of 2 *N* HCl and the resulting suspension was immediately centrifuged and the precipitate was discarded. The supernatant was quickly adjusted to pH 7.0 by addition of 2 *N* NaOH. Solid ammonium sulfate was added to the above enzyme solution and a fraction precipitating between 35 and 70% saturation was collected. The precipitate was dissolved in water and dialysed for 20 hours against running distilled water. The dialysed solution was treated with two volumes of chilled acetone (−15°C) and the precipitate was centrifuged, taken up in water, and centrifuged again. The clear, light yellow-colored supernatant was quickly frozen and lyophilized. This was used as lyophilized preparation. A typical purification procedure and the hydrolyzing activity for palmitoyl-DL-valine as substrate were summarized in Table I.

The acetone-dried cells and the lyophilized preparation had a stable hydrolytic activity against all the tested acyl-amino acids for at

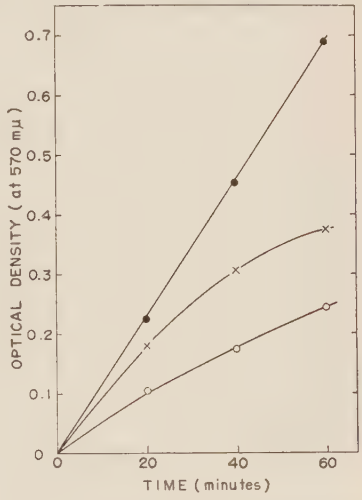

FIG. 1. Hydrolysis of N-palmitoyl-amino acids as a function of time.

The assay system was as described in the text. The lyophilized preparation (34 μg. of protein N) was used as enzyme. —×—: 0.1 ml. of 0.1 *M* palmitoyl-DL-valine, —○—: 0.2 ml. of 0.025 *M* palmitoyl-L-phenylalanine, —●—: 0.1 ml. of 0.05 *M* palmitoyl-L-aspartic acid.

TABLE I

Summary of Purification Procedure of the Enzyme Hydrolysing N-Palmitoyl-DL-Valine

Stage of purification	Total volume (ml.)	Total protein N (mg.)	Total activity (units)	Specific activity (units/mg. of protein N)	Yield (%)
Crude extract	200	630	945	1.5	100
Acid precipitation	200	280	749	2.7	79
Ammonium sulfate fractionation	200	110	541	4.9	57
Acetone precipitation	71	72	370	5.1	39
Lyophilization	250	28	188	6.7	20

1 Unit = 1 μmole of free amino acid formed/hour.

least six months when stored in desiccator *in vacuo*. The lyophilized preparation was dissolved in 0.02 *M* phosphate buffer or water, usually at an amount of 2 mg. of preparation per ml. of solvent, prior to each experiment.

The Enzymatic Hydrolysis of N-Palmitoyl-Amino Acids—The hydrolysis of the palmitoyl-amino acids proceeded as a function of time for first 40 minutes as illustrated in Fig. 1, and the relations between the hydrolytic activity and the enzyme quantity were proportional as given in Fig. 2.

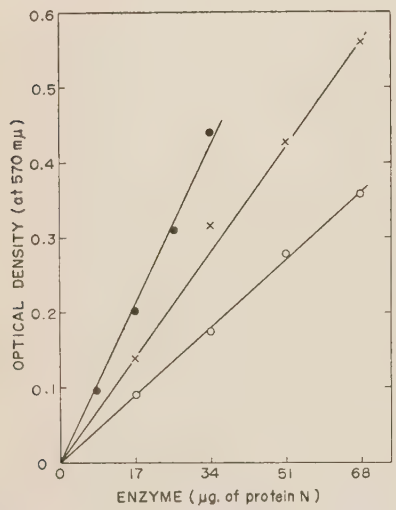

FIG. 2. Rate of hydrolysis of N-palmitoyl-amino acids as a function of enzyme concentration.

The assay system was the same as Fig. 1. Incubation was for 40 minutes. —×—: palmitoyl-DL-valine, —○—: palmitoyl-L-phenylalanine, —●—: palmitoyl-L-aspartic acid.

The Hydrolyzing Activities of the Lyophilized Preparation for Various N-Acyl-Amino Acids—The lyophilized preparation hydrolyzed all the tested acyl-amino acids as shown in Table II.

The effects of varying concentrations of the acyl-amino acids on the reaction rate were determined, and Fig. 3 shows the cases of palmitoyl-amino acids. The maximal activitity was attained at the substrate concentration of 1×10^{-3} *M* and was maintained at least up to 5×10^{-3} *M* of the substrate, in contrast with the report of Mounter *et al.*

TABLE II

Hydrolytic Activities of Lyophilized Preparation for Various N-Acyl-Amino Acid

Substrate	Activity (μmoles/hr./mg. of protein N)
Palmitoyl-DL-valine	31.0
Palmitoyl-L-phenylalanine	27.9
Palmitoyl-L-aspartic acid	63.1
Acetyl-DL-valine	22.5
Chloroacetyl-L-phenylalanine	2.3
Acetyl-L-aspartic acid	5.9

The condition was as described in the text. The amount of enzyme was 17 μg. of protein *N*.

(*13*) who found that the substrate inhibition increased as the substrate concentration increased in hydrolysis of the acetyl-, propionyl-, and butyryl-glycine by the hog kidney acylase I. The Michaelis constants, calculated from Lineweaver and Burk's plot, of all the tested acyl-amino acids were indicated in Table III.

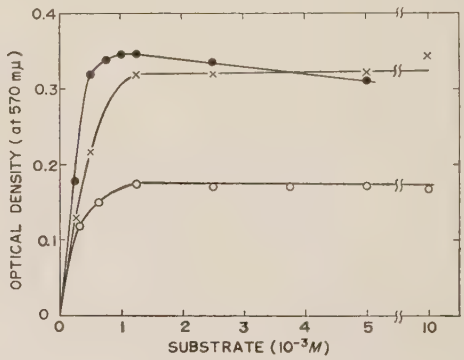

FIG. 3. Effect of substrate concentration on hydrolysis of N-palmitoyl-amino acids.

Incubation was for 40 minutes. Lyophilized preparation (34 μg. of protein N) was used. —×—: palmitoyl-DL-valine, —○—: palmitoyl-L-phenylalanine, —●—: palmitoyl-L-aspartic acid.

The effect of pH on the hydrolyzing activity for the various acyl-amino acids was shown in Fig. 4. The pH optima were as follows: pH 7.4 for palmitoyl-DL-valine and palmitoyl-L-phenylalanine; pH 6.2 for palmitory-L-aspartic acid; pH 7.0 for acetyl-DL-valine; pH 7.1 for chloroacetyl-L-phenylalanine; and pH 6.0 for acetyl-L-aspartic acid.

Table III

Michaelis Constants for Various N-Acyl-Amino Acids

Substrate	K_M (M)
Palmitoyl-DL-valine	4.8×10^{-4}
Palmitoyl-L-phenylalanine	3.0×10^{-4}
Palmitoyl-L-aspartic acid	2.0×10^{-4}
Acetyl-DL-valine	1.0×10^{-3}
Chloroacetyl-L-phenylalanine	4.2×10^{-4}
Acetyl-L-aspartic acid	4.4×10^{-3}

The condition was as described in the text, except that 0.1 M citric acid-0.2 M phosphate buffer, pH 6.1, was used for acetyl-L-aspartic acid.

The effect of metal ions on the hydrolyzing activities was shown in Table IV. Co^{++} accelerated the hydrolysis of palmitoyl-DL-valine and chloroacetyl-L-phenylalanine, and Zn^{++}, Hg^{++} and Cu^{++} were inhibitory on the breakdown of some substrates. But it should be noted that Cu^{++} catalyzed the non-enzymatic hydrolysis of the substrates except for palmitoyl-L-aspartic acid.

The heat stability of the activities hydrolyzing the acyl-amino acids was illustrated

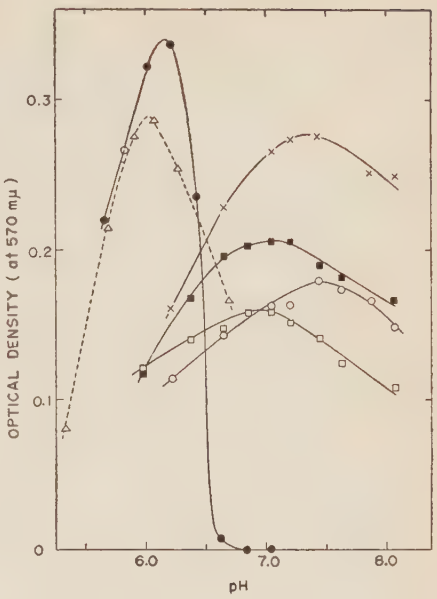

Fig. 4. Effect of pH on hydrolysis of various N-acyl-amino acids.

—: 0.2 M phosphate buffer, ---: 0.1 M citric acid-0.2 M phosphate buffer. —×—: palmitoyl-DL-valine, —○—: palmitoyl-L-phenylalanine, —●—: palmitoyl-L-aspartic acid, —□—: acetyl-DL-valine, —■—: chloroacetyl-L-phenylalanine, —△—: acetyl-L-aspartic acid.

Table IV

Effect of Metal Ions on the Hydrolyzing Activities for Various N-Acyl-Amino Acid

Substrate	Metal (M)							
	Co^{++}		Zn^{++}		Cu^{++}		Hg^{++}	
	1×10^{-4}	1×10^{-3}	1×10^{-4}	1×10^{-3}	1×10^{-4}	1×10^{-3}	1×10^{-5}	1×10^{-4}
	Activity (%)							
Palmitoyl-DL-valine	124	209	119	144	94	0	84	98
Palmitoyl-L-phenylalanine	123	36	117	0	83	0	93	94
Palmitoyl-L-aspartic acid[1)]	111	66	66	54	94	73	90	0
Acetyl-DL-valine	89	101	85	45	58	27	12	10
Chroroacetyl-L-phenylalanine	143	192	48	23	5	0	0	0
Acetyl-L-aspartic acid	92	73	21	46	4	0	0	0

The condition was as described in the text, except that 0.2 M Tris buffer, pH 7.1, was used for acyl-valine and -phenylalanine; 0.2 M phosphate buffer, pH 6.1, for palmitoyl-aspartic acid; and 0.2 M succinate-NaOH buffer, pH 6.0, for acetyl-aspartic acid.

Control values without metal ions were taken as 100% activity.

1) The activity for palmitoyl-L-aspartic acid required the existence of phosphate buffer.

in Fig. 5. After 10 minutes incubation at 90°C, the hydrolytic activities for the acetyl- or chloroacetyl-amino acids were completely lost, but the activities hydrolyzing the palmitoyl-amino acids remained unaffected at least in 60 per cent. This fact suggests that the enzymes catalyzing the hydrolysis of palmitoyl-amino acids are different from the enzymes hydrolyzing acetyl- or chloroacetyl-amino acids.

In the experiment of animal organs, Izar (*3*) reported that the activities to hydrolyze his long chain acyl-amino acids were lost by heating at 56–7°C for 30 minutes.

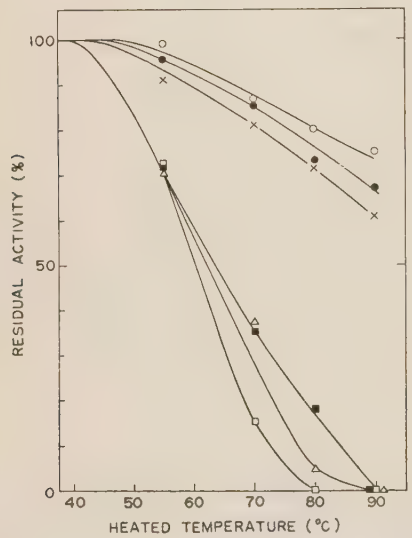

FIG. 5. Heat stability of enzymes hydrolyzing N-acyl-amino acids.

The enzyme solution in 0.02 *M* phosphate buffer, pH 7.0, was incubated for 10 minutes at each temperature. The residual activities for various acyl-amino acids were assayed as described in the text, except that for acetyl-aspartic acid 0.5 ml. of 0.1 *M* citric acid-0.2 *M* phosphate buffer, pH 6.1, was used. Control assays were carried out for each experiment. Control values were taken as 100 per cent activity.

—×—: palmitoyl-DL-valine, —○—: palmitoyl-L-phenylalanine, —●—: palmitoyl-L-aspartic acid, —□—: acetyl-DL-valine, —■—: chloroacetyl-L-phenylalanine, —△—: acetyl-L-aspartic acid.

According to Fukui and Axelrod (*7*), rat liver preparation was active for the synthesis and the hydrolysis of palmitoyl-L-phenylalanine and the crystalline carboxypeptidase also catalyzed the breakdown of this substrate. In this respect the author tested the hydrolyzing activity of various acyl-amino acids using a highly purified Taka-acylase. As shown in Table V, the

TABLE V

Hydrolytic Activities of Taka-Acylase Preparation for Various N-Acyl-Amino Acids

Substrate	Activity (μmoles/hr./mg. of protein N)
Palmitoyl-DL-valine	12.7
Palmitoyl-L-phenylalanine	0.0
Palmitoyl-L-aspartic acid	0.7
Acetyl-DL-valine	1374.7
Chloroacetyl-L-phenylalanine	1847.0
Acetyl-L-aspartic acid	131.5

The condition was as described in the text, except for coexistence of 5×10^{-4} *M* $CoCl_2$.

preparation had a high hydrolyzing activity for acetyl- or chloroacetyl-amino acids, while palmitoyl-amino acids were not or hardly attacked by the preparation.

DISCUSSION

The lyophilized preparation, extracted and partially purified from *Mycobacterium avium*, had hydrolyzing activities for N-palmitoyl-amino acids. Some different properties can be seen in the hydrolytic activity to each substrate, especially between palmitoyl- and acetyl-DL-valine, between palmitoyl- and chloroacetyl-L-phenylalanine, and between palmitoyl- and acetyl-L-aspartic acid. By the experiment of heat stability, it seems that these hydrolytic enzymes for palmitoyl-amino acids are clearly different from the hydrolyzing enzymes for acetyl- or chloroacetyl-compounds in which amino acid component was the same to that of the palmitoyl-amino acid.

On the problem whether the hydrolysis of palmitoyl-valine, -phenylalanine, and -aspartic acid was catalyzed by the same enzyme, the answer should be made by the

experiments using a higher purified preparation. Birnbaum (*1*) has classified the acylase of hog kidney into acylase I, II and III, but in the experiment of Taka-acylase the acetyl- or chloroacetyl-derivatives of valine and aromatic amino acids seemed to be hydrolysed by a single enzyme (*14*). In this report, it is suggested that the enzyme hydrolyzing palmitoyl-L-aspartic acid is to be distinguished from the enzymes for the other two palmitoyl-amino acids in respect to the different susceptibility to pH.

Recently, Fukui and Axelrod (*7*) studying the synthesis of lipo-amino acid, found that the rat liver preparation catalyzed the formation of phenylalanine-lipid compounds from free oleic or palmitic acid more effectively than from glycerides. It should be noted that in cholinesterase of animal organs, the enzyme catalyzing hydrolysis of palmitoyl-choline also differed from the so far known cholinesterases (*15*).

SUMMARY

The enzymes catalyzing the hydrolysis of N-palmitoyl-DL-valine, N-palmitoyl-L-phenylalanine, and N-palmitoyl-L-aspartic acid, besides N-acetyl-DL-valine, N-chloroacetyl-L-phenylalanine, and N-acetyl-L-aspartic acid, have been extracted and partially purified from *Mycobacterium avium*.

In studies on the properties of the hydrolyzing activities, it was evidenced that the enzymes for N-palmitoyl-amino acids were distinguished from the enzymes for N-acetyl- or N-chloroacetyl-amino acids.

The author wishes to express his sincere gratitude to Prof. Akabori of Osaka Univ. and Prof. Yamamura of Kyushu Univ. for their constant interest and encouragement.

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Studies on Habu Snake Venom

IV. Fractionation of Habu Snake Venom by Chromatograpy on CM-Cellulose

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Since the discovery that snake venoms hydrolyze phosphodiester bonds, both in polyribonucleotides and polydeoxyribonucleotides (*1*, *2*), a number of fractionation procedures have been worked out for the purification of venom phosphodiesterase and its separation from 5'-nucleotidase (*3–6*). Boman *et al.* (*6*) have recently demonstrated that the phosphodiesterase in the venom of a rattle snake, *Crotalus adamanteus*, was heterogeneous and that all the enzyme fractions isolated were active toward DNA. The hypothesis by Laskowsky *et al.* (*7*) that the diesterase might be an exonucleotidase acting on the 5'-phosphoester bond of a chain and removing 5'-mononucleotides by successive attack, has been verified by Razzel and Khorana (*8*). More recently Suzuki and Iwanaga (*9*) also found three phosphodiesterases in the venom of Mamushi (*Agkistrodon blomhoffi*), and demonstrated that none of these active fractions was artificially formed during the collection of the venom, as indicated in the case of a rattle snake venom by Boman *et al.* (*10*). On the other hand, Taborda (*11*, *12*) and Crestfield (*13*) suggested the existence of RNase and DNase, although their isolation was not carried out.

In spite of these abundant enzymatic studies on snake venoms, very poor are the knowledge about the biochemical mechanisms whereby the pathological changes are produced. The clinical symptoms of Habu bite are divided into two groups: general symptoms and local singns (*14*). Mitsuhashi and Maeno *et al.* (*14–17*) demonstrated that proteolytic enzymes are one of the principles which cause myolysis in Habu bite. Recently, Maeno *et al.* (*18*) demonstrated that phospholipase A is also responsible for the myolysis caused by Habu bite.

The present report deals with the chromatographic separation of the venom of Japanese venomous snake, Habu (*Trimeresurus flavoviridis*). It is worthy of record that more than one type of nuclease activity containing that of RNase have been found in Habu venom. Lethal activities were also investigated in relation to enzymes in the venom.

MATERIALS AND METHODS

The freeze-dried venom from Habu, *Trimeresurus flavoviridis*, was kindly supplied by Dr. Y. Sawai, the Institute for Infectious diseases, University of Tokyo. Yeast RNA and thymus DNA were purchased from the Minophagen Pharmaceutical Manufacturing Co. LTD. Adenosine 5'-phosphate, calcium salt of bis (*p*-nitrophenyl) phosphate and *p*-nitrophenyl phosphate were the products of Sigma Chemical Company. The activities of both RNase and DNase were assayed according to the method of Sato and Egami (*19*) by determination at 260 mμ of the uranyl acetate filtrate with a Beckman model DU spectrophotometer. The reaction mixture containing 0.1 ml. of enzyme solution, 0.1 ml. of 10^{-2} *M* $MgCl_2$, 0.5 ml. of RNA (6 mg./ml. of 0.1 *M* Glycine-NaOH buffer, pH 9.0) and 0.3 ml. of water was incubated at 37°C for 30 minutes for the assay of RNase and 60 minutes for the assay of DNase. The reaction was stopped by the addition of uranyl reagent. For the measurement of the extent of digestion the uranyl acetate filtrate was determined spectrophotometrically at 260 mμ. 5'-Nucleotidase activity was determined by the method

The abbreviations used are: DNA, doexyribonucleic acid; DNase, deoxyribonuclease; DNPP, bis (*p*-nitrophenyl) phosphate; EDTA, ethylenediamine tetraacetic acid; PCMB, *p*-chloromercuribenzoate.

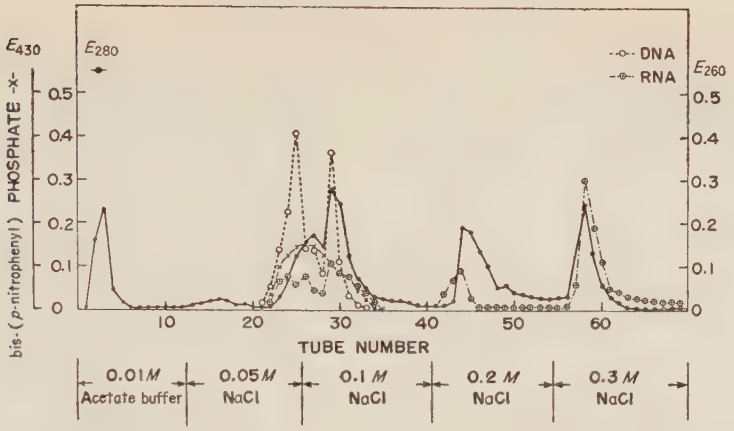

FIG. 1A. Distribution of proteins, phosphodiesterase, DNase and RNase separated by column chromatography on CM-cellulose. The protein concentration of each fraction with ten fold dilution was determined spectrophotometrically at 280 mμ. An aliquot of each fraction was used for the determination of enzyme activities.

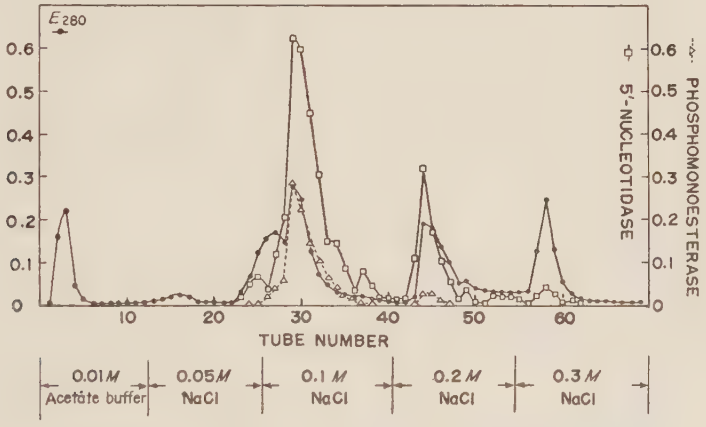

FIG. 1B. Distribution of proteins, 5'-nucleotidase and phosphomonoesterase separated by the same chromatography as in Fig. 1A.

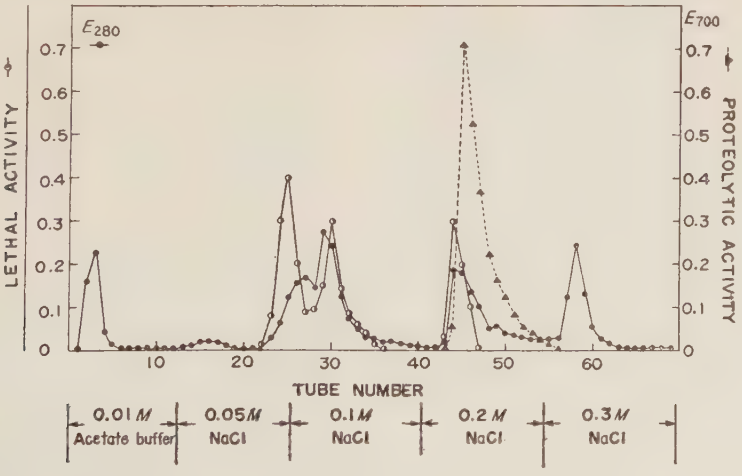

FIG. 1C. Distribution of proteins, proteolytic enzyme and lethal toxicity separated by the same chromatography as in Fig. 1A.

of Private de Garilhe *et al.* (*5*), using Allen's method (*20*) for the determination of inorganic phosphate. The reaction mixture containing 0.2 ml. of enzyme solution, 0.1 ml. of 10^{-2} *M* $MgCl_2$, 0.4 ml. of 0.1 *M* veronal buffer, pH 8.6 and 0.3 ml. of 0.01 *M* adenosine 5'-phosphate was incubated at 37°C for 30 minutes.

Phosphodiesterase was determined by the method of Sinsheimer and Koerner (*21*). The incubation mixture contained 0.1 ml. of enzyme solution, 0.5 ml. of 0.1 *M* veronal buffer, pH 9.0, 0.35 ml. of 10^{-2} *M* $MgCl_2$, 2.0 ml. of 10^{-3} *M* DNPP. The total volume was made to 3.5 ml. by the addition of water. After 15 minutes' incubation at 37°C the reaction was stopped by the addition of 2 ml. of 2 *N* NaOH and immediately determined spectrophotometrically at 430 mμ. For the measurement of phosphomonoesterase activity, the reaction mixture containing 0.5 ml. of enzyme solution, 0.5 ml. of 0.1 *M* veronal buffer, pH 7.4, 0.2 ml. of 10^{-2} *M* $MgCl_2$, 0.5 ml. of 0.01 *M* *p*-nitrophenyl phosphate and 0.55 ml. of water was incubated for 60 minutes at 37°C. The reaction was stopped by the addition of 10% NaOH and the *p*-nitrophenol released was determined spectrophotometrically at 430 mμ.

Proteinase activity was measured according to the method described in the previous paper (*15*).

Lethal toxicity was assayed by an intraperitoneal injection of serial dilutions of each fraction with 0.1 to 0.2 ml. into mice weighing about 14 to 15 g. Four mice were used for each dilution. All deaths during 24 hours following injection were ascribed to venom toxicity.

The protein content was expressed as the optical density at 280 mμ using a Beckman model DU spectrophotometer with a cell of 1 cm. light path. A solution containing 100 μg. protein per ml. in crude venom was found to have an optical density of 0.156.

In the chromatographic technique carboxymethyl (CM) cellulose from Serva was used as adsorbent. The experiment was performed with 200 mg. of the venom on a column (1.6×15 cm). Before applying the venom solution, the CM cellulose was equilibriated with 0.01 *M* acetate buffer, pH 6.0 and it was always checked whether the effluent from the column had the correct pH. After equilibrating the column, the elution was performed with various concentrations of NaCl containing 0.01 *M* sodium acetate at a flow rate of 20 ml. per hour, and 6.0 ml. fractions were collected by a fraction collector. All the experiments in chromatographic technique were carried out at 2°C.

RESULTS

Chromatography of Phosphodiesterases—Fig. 1A shows the chromatographic behaviour of the diesterase activities on the DNPP, DNA and RNA as substrates used.

The pattern of distribution of diesterases, using RNA as substrate showed that at least four diesterases are present in Habu venom and that a half of the diesterase activities was recovered in 0.3 *M* NaCl effluent fraction and the others distributed in 0.05 *M*, 0.1 *M* and 0.2 *M* NaCl effluents, respectively. The fact that diesterases in both 0.2 *M* and 0.3 *M* NaCl effluents are not active on DNA and DNPP indicates that both diesterases are RNase and they are designated as RNase H2 and RNase Hl, respectively. As shown in Figs. 1A and B, both RNases contain 5'-nucleotidase. The result of the purification of RNase Hl is given in Table I where the preparation with only 4 per cent of the total 5'-nucleotidase content in the crude venom was four fold purified and its recovery was approximately 50 per cent.

TABLE I

RNase Hl Activity Separated by Column Chromatography on CM-Cellulose

RNase activity was expressed by the optical density at 260 mμ of the uranyl acetate filtrate as described in the text. Tube No. in this table is the same as in Fig. 1A.

Fractions	Total protein	Tatal activity	Specific activity	Activity recovered
	mg.	E_{260}	E_{260}/mg.	%
Crude venom	200	0.230×10^3	1.25	100
Tube No. 57—62	23	0.113×10^3	4.19	49

The contaminated 5'-nucleotidase in the preparation is successfully removed by rechromatography. The other enzyme activities found in Habu venom such as protease, phospholipase A, L-amino acid oxidase, phosphomonoesterase as well as biological activities such as hemorrhagic and lethal activities are all removed. Details on the enzymatic properties of RNase Hl will be reported in the following paper (*22*).

TABLE II

Phosphodiesterase Activity in 0.05M NaCl Effluent Fraction in Fig. 1

Phosphodiesterase activity using DNPP or DNA as substrate is expressed by the optical density at 430 mμ or 260 mμ formed in the assay conditions described in the text. Tube No. in this table is the same as in Fig. 1.

Fraction	Total protein	Phosphodiesterase						Relative activity of 5'-nucleotidase
		DNPP			DNA			
		Total activity	Specific activity	Activity recovered	Total activity	Specific activity	Activity recovered	
	mg.	E_{430}	E_{430}/mg.	%	E_{260}	E_{260}/mg.	%	
Crude venom	200	0.160×10^3	0.80	100	0.38×10^3	1.90	100	100
Tube No. 23—26	14.6	0.310×10^2	2.12	19.4	0.11×10^3	7.50	28.9	4

The measurement of diesterase activities of each fraction, using DNPP and DNA as substrate shows that they are active in 0.05 *M* and 0.1 *M* effluent fractions. In 0.1 *M* NaCl effluent were found potent activities of L-amino acid oxidase, monoesterase, phospholipase A and 5'-nucleotidase, which were quite difficult to be separated from diesterase, even with rechromatography by CM-cellulose and zone electorphoresis using starch as supporting medium. For further purification of diesterase it is, therefore, convenient to employ the first peak corresponding to the tubes 23–26 in Fig. 1A, containing only 4 per cent of the total 5'-nucleotidase content in Habu venom. Table II shows that the diesterase using DNA as substrate is four fold purified, whereas diesterase using DNPP is 2.7 fold purified. In addition, both activities do not parallel with each other in column chromatographic behaviour as shown in Fig. 1A.

These results will suggest the presence of two enzymes differing in the extent of hydrolysis of DNA or DNPP. The pooled preparations obtained in the same two experiments were resubmitted to chromatography after dialyzing against water at 2°C under the same conditions described. The rechromatogram of the combined fractions corresponding to the tubes 23–26 is given in Fig 2. The preparations thus obtained are still contaminated with less than 1 per cent of the total 5'-nucleotidase in the crude venom.

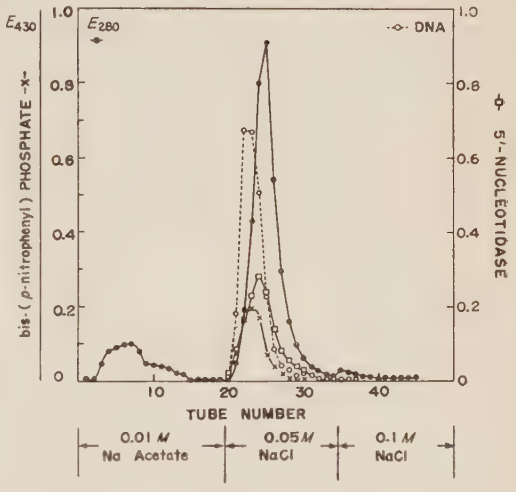

FIG. 2. Distribution of proteins, DNase, phosphodiesterase and 5-nucleotidase separated by rechromatography of LF I on CM-cellulose. The protein concentration of each effluent was determined spectrophotometrically at 280 mμ.

Diesterase activity in the preparation was completely inhibited by EDTA at the concentration of 10^{-3} *M*, whereas RNase HI was more or less activated by EDTA.

Proteolytic Enzymes—It was already reported that there were found at least three proteolytic enzymes in Habu snake venom (*15–17*, *23*). From the column chromatographic studies as seen in Fig. 1C it was found that only in 0.2 *M* NaCl effluent fraction was observed proteolytic activities, but not in other effluent fractions.

Lethal Toxicities with Their Relations to Other Enzyme Activities—The result of toxicity tests on each chromatographic fraction is shown in Fig. 1C. It is obvious that the venom contains at least three lethal fractions, designated as LF I, II and III corresponding to tube No. of their activity peaks, 25, 30 and 44, respectively, for the sake of convenience. LF I and II are completely separated from proteolytic enzymes, whereas LF III is coexistent with the proteinase fraction. However, the chromatographic pattern of LF III and proteolytic activities in Fig. 1C will suggest that they are separable from each other. The peak of the 5'-nucleotidase runs parallel to that of LF III. The fact that LF I runs parallel to DNase activity strongly suggested that DNase will be responsible for a lethal factor as seen in Fig. 1A and C. However, the results of the rechromatography of the fraction in Fig. 2 and Table III show that they are also separable from each other. Lethal toxicity of each fraction separated by rechromatography of LF I is given in Table III. LF II was also separable from all other enzymes except 5'-nucleotidase investigated.

Table III

Lethal Toxicities of Each Fraction Separated by Rechromatography of LF I

After dialyzing against distilled water with mechanical shaking for several hours at 2°C, LF I was resubmitted to chromatogram on CM-cellulose under the same conditions described in the text. One tenth ml. of each fraction was intraperitoneally injected into mice. Each test group constitued of four animals.

Tube. No. (See Fig. 3)	No. of death	
	without dilution	with two fold dilution
21	0	0
22	2	0
23	4	2
24	4	3
25	4	2
26	2	0
27	0	0

Discussion

Phosphodiesterase—It is noteworthy that from the studies on the column chromatographic separation of Habu venom by CM-cellulose two RNase designated as RNase H1 and H2 were found in the venom. Maeno and Mitsuhashi (*22*) reported that RNase H1 shows two optimal pHs, 8.3 and 8.9 and that hydrolysis of yeast RNA by RNase H1 gives the "RNase-resistant" fraction. Taborda *et al.* (*11*, *12*) suggested the presence of DNase and RNase from the fact that a rattle snake venom can hydrolyze highly polymerized DNA and RNA. Laskowsky *et al.* (*7*) suggested the presence of DNase in a rattle snake venom from the result that the activity decreasing the viscosity of DNA by the venom was highest in acid pHs, quite different from the optimal pH of diesterase. In spite of the suggestions by these workers isolation of the enzymes were not performed. It will suggest the presence DNase in Habu venom in addition to RNase that diesterase activity using DNA as substrate does not parallel with that using DNPP as shown in Fig 1A. From the data it can be reasonably assumed that at least more than five diesterase containing DNase, RNase H1 and H2, are present in Habu venom. It was also demonstrated that Habe venom gave at least two chromatographically separable fractions, both of which have 5'-nucleotidas activity. Ohsaka (*24*) has demonstrated that Habu venom gave a 5'-nucleotidase and a phosphodiesterase fraction by zone electrophoresis at pH 9.2 using starch as supporting medium.

Proteolytic Activity—Maeno *et al.* already found at least three proteolytic enzymes in Habu venom, optimal pHs of which were 7.3, 8.3 and 8.9, respectively (*15–19*, *23*). The similar result was obtained by Ohsaka (*25*) that at least five electrophoretic components of proteolytic activity, were present in the venom and could be categolized at least three distinct enzymes. However, it was failed to separate successfully these proteolytic enzymes by the column chromatographic technique.

Relations of Lethal Activity to Other Enzyme Activities—Colum chromatographic separation of Habu venom on CM-cellulose revealed that three components of lethal activity were present in the venom designated as LF I, II and III, all of which were separable from main peaks of DNase, RNase, diesterase, monoesterase and proteinase. Similar results were obtained by Ohsaka (*24*) by zone electrophoretical fractionation. The peak of the lethal activity in LF I was idential with a very small peak of 5'-nucleotidase and the rechromatography of the fraction gave also the same results as seen in Fig. 2 and Table III. However, it is in doubt that this minor part of the 5'-nucleotidase is responsible for lethal toxicity in Habu vonom. According to Zeller (*26*) 5'-nucleotidase may be responsible for the production of fatal shock. None of enzymes are so far presumably responsible for lethal toxicity, although the possibility that the minor part of 5'-nucleotidase might be a principle of lethal toxicity can not be ruled out.

SUMMARY

The column chromatography of a venom of Japanese venomous snake, Habu (*Trimeresurus flavoviridis*), on carboxymethyl cellulose revealed that at least five diesterases containing two RNases and one DNase are present, all of which were separable from lethal toxicity.

Phosphomonoesterase and proteolytic enzymes may also be separable from the toxicity.

There were found at least three 5'-nucleotidase fractions which were chromatographically separable from each other. Two of these nucleotidase fractions run parallel to lethal toxicity.

The authors are indebted to Prof. F. Egami, Department of Biophysics and Biochemistry, the University of Tokyo, for his helpful advice.

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Electron Transporting Components Participating in Nitrate and Oxygen Respirations from a Halotolerant *Micrococcus*

I. Purification and Properties of Cytochromes b_4 (I) and b_4 (II)

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Recently, several different cytochromes of type-b which might participate both in oxygen and nitrate reducing systems have been isolated from denitrifing bacteria, *Micrococcus denitrificans*, *Pseudomonas denitrificans* (*1*), *Pseudomonas aeruginosa* (*2*) and also from *Escherichia coli* (*3*).

Many investigators have suggested that in *Micrococcus denitrificans* and *Pseudomonas aeruginosa* cytochrome of type "c" was also involved in nitrate reducing system (*4–7*). A halotolerant strain of *Micrococcus* has been shown to contain a cytochrome which was designated as cytochrome b_4 (*8*). Thereafter, the cytochrome was shown to consist of two spectroscopically distinct components; in reduced form, one of these cytochromes had double α-bands and the other had a single α-band. They were tentatively named as cytochrome b_4 (type I) and cytochrome b_4 (type II), respectively (*9*).

It was also reported from this laboratory that the same organism grown aerobically in the presence of nitrate contained NaR*, NiR and HdR (*10–12*).

Recently, however, this *Micrococcus* sofar accepted as an aerobe has been shown to be successfully grown anaerobically by the addition of sodium formate to the culture medium (*13*). From the *Micrococcus* grown under aerobic and anaerobic conditions, five chromoproteins including HdR, cytochromes b_4 (I) and b_4 (II) have been obtained in soluble forms and each was purified by column chromatography on DEAE-cellulose. The present report deals with the methods for purification of these five chromoproteins and with properties of cytochromes b_4 (I) and b_4 (II).

* Abbreviations: NaR, nitrate reductase; NiR, nitrite reductase; HdR, hydroxylamine reductase; DPNH, reduced diphosphopyridine nucleotide; DEAE, N, N'-Diethylaminoethyl.

MATERIALS AND METHODS

Bacterial Culture—A halotolerant denitrifying *Micrococcus*, strain No. 203 was used. Aerobic cells were grown in the following basal medium into which air was bubbled continuously. Basal medium: NaCl 100g., KH_2PO_4 10g., $MgSO_4 \cdot 7H_2O$ 0.2g., yeast extract powder 2g., meat extract 10g., polypeptone 10g. and 10mg. of heavy metal mixture ($FeSO_4$, $CuSO_4$ and $MnCl_2$), pH 7.2 per liter of tap water. Anaerobic cells were grown in the basal medium enriched with 1 per cent of KNO_3 and 1 per cent of sodium formate. The cultivation was carried out in a large quantity in a stainless steel tank*(100 liters) at 35°C for 24 hours and the cells were harvested by Sharples centrifuge and were washed two or three times with cold 10% sodium chloride. Washed cells were then lyophilized (2 kg. of dry cells were obtained)

Extraction of Chromoproteins—Lyophilized cells (200 g.) were suspended in a concentration of 80 to 100mg. per ml. in 0.01 *M* sodium phosphate buffer, pH 6.8, which contained 50 μg. per ml. of DNase and the suspension was dialyzed over night against the same buffer. Unless otherwise indicated, all procedures were carried out at 4°C. After dialysis, the cell suspension was centrifuged at $5{,}000 \times g$ for 30 minutes to remove insoluble materials and cell debris. The

* The cultivation of aerobic and anaerobic cells of this *Micrococcous* was carried out of courtesy of Nagoya Factory of Fujisawa Chemicals Co. Ltd.

extracts thus obtained were centrifuged at 20,000×*g* for 30 minutes to remove the pale reddish precipitate which contained mainly subcellular particles having activities of NaR and cytochrome oxidase, and the particulate factor of NiR (*11*) in addition to particule bound cytochromes b_4 (I), c^{551} and genuine cytochrome of b-type. The precipitate was washed twice with same buffer, the washings were combined and added to the brownish red supernatant fluid. Sodium phosphate buffer of pH 6.8 and sodium chloride were then added to the supernatant in final concentrations of 0.02 *M* and 0.3 *M*, respectively. Chilled acetone was then added dropwise to the supernatant with vigorous stirring. The precipitate obtained between 40 and 75% (v/v) acetone was collected and dissolved in a minimal volume of distilled water and dialyzed for 8 to 10 hours against distilled water (2 or 3 changes) and lyophilized. Cytochromes b_4 (I) and c^{551} bound to subcellular particles were extracted with 40% (v/v) acetone and fractionated by the same procedure as mentioned above.

Physical and Chemical Determinations—1. Sedimentation constant, diffusion constant and molecular weight of cytochrome b_4 (I) and sedimentation constant of cytochrome b_4 (II) were measured by Prof. N. Ui of Gunma University.

2. Measurements of electrophoretic mobility were carried out using Hitachi electrophoresis apparatus (Tiselius type) at 2.4°C.

3. Pyridine hemochromogens were prepared according to the method of Vernon and Kamen (*14*).

4. Hydrochloric acid-methylethylketone treatment were followed in the method of Teale (*15*).

5. Oxidation-reduction potentials were measured at 25°C using ferri-ferro oxalate system according to the directions of Velick and Strittmatter (*16*).

6. Iron analyses were performed by the *o*-phenanthroline method of Sandel (*17*) with some modifications in which hydrogen peroxide and fuming nitric acid in stead of concentrated sulfuric acid and concentrated nitric acid were used for ashing of these cytochromes.

7. All spectroscopic measurements were made by means of a Hitachi model EPU-2 Spectrophotometer using 1 cm. cells.

8. Spectroscopic observation of cytochromes in cell suspensions was performed by Prof. T. Mori, Department of Biology, Nagoya University using a Hartridge hand spectroscope with usual method.

9. Protein was determined by the method of Lowry *et al.* (*18*).

Sources of Chemicals—1. DEAE-cellulose was obtained from Eastman Organic Chemicals.

2. Crystalline DNase was a gift of Dr. T. Nozima of Institute for Infectious Diseases, University of Tokyo.

3. DPNH (87% purity), Cytochrome c(horse heart, 70% purity) were obtained from Sigma Chemical Company.

4. All other reagents were of reagent grade.

RESULTS

Chromatographic Separation and Purification of Chromoproteins—The lyophilized preparation was dissolved in 0.1 *M* ammonium acetate buffer, pH 6.0, and dialyzed against the same buffer for several hours. The dialysate was then centrifuged to remove insoluble materials, when necessary. The clear brownish red solution thus obtained was passed through a 4×15 cm. DEAE-cellulose column which had been equilibrated with 0.1 *M* ammonium acetate buffer, pH 6.0. Chromoproteins were completely adsorbed as a band on the upper part of the column. After the solution had been applied to the column, it was washed with 0.1 *M* and 0.15 *M* ammonium acetate buffer, pH 6.0 successively. By this treatment most of colorless proteins adsorbed were eluted from the column without disturbing the colored band. Thereafter, as is shwon in Fig. 1, chromoproteins were eluted separately from the column by the stepwise increase of concentrations of ammonium acetate buffer, pH 6.0; with 1.7 *M* of the buffer a pale reddish band (cytochrome c^{551}) began to move which was eluted out by 0.2 *M* of the same buffer. By 0.3 *M* of the buffer a brown protein was completely eluted out. Following the above chromoproteins, cytochromes b_4 (I), b_4 (II) and HdR (cytochrome 625, 553) were eluted by 0.4 to 0.45 *M*, 0.5 to 0.6 *M* and 0.60 to 0.65 *M* ammonium acetate buffer, pH 6.0 respectively.

These fractions obtained were diluted with distilled water until the concentration of ammonium acetate buffer in each eluate became lower than 0.1 *M* and then rechromatographed on separate DEAE-cellulose column by the same procedure as mentioned above.

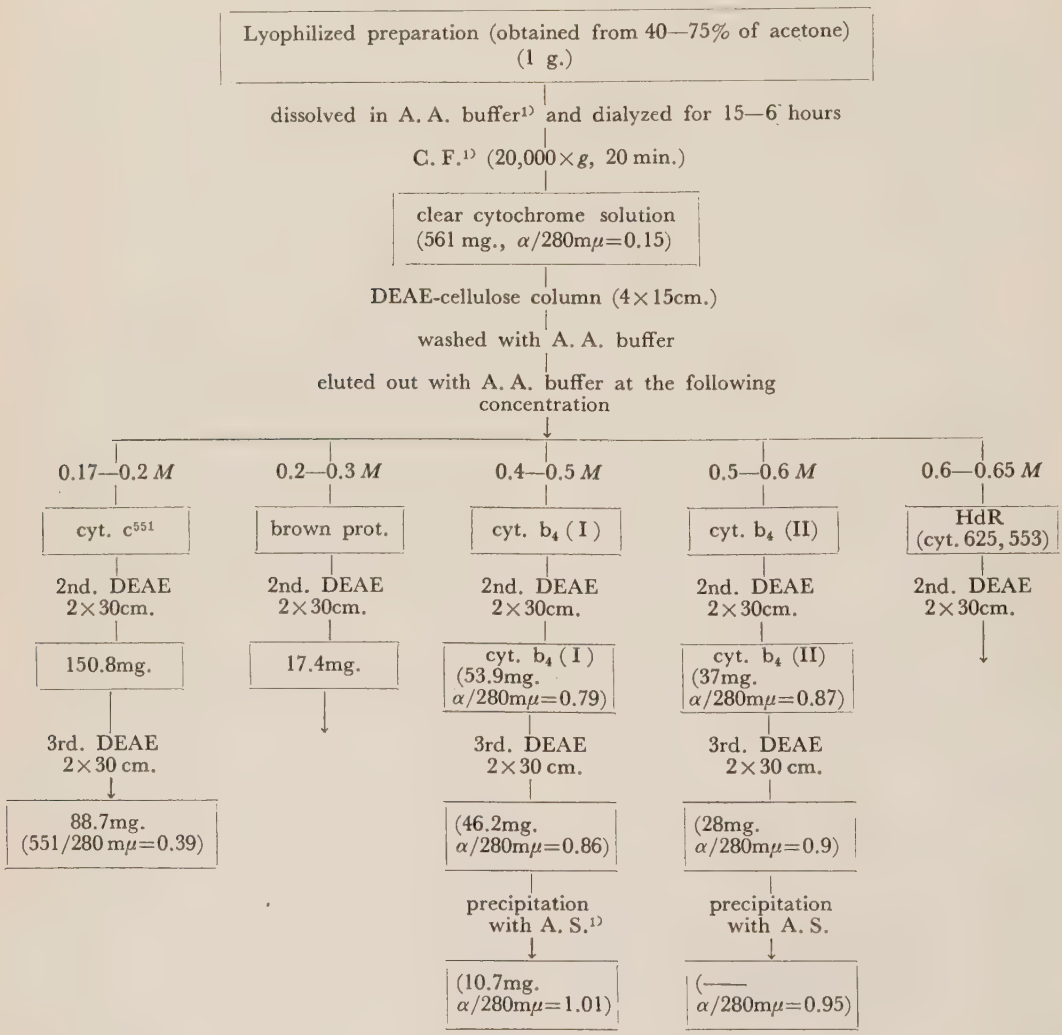
Each chromoprotein obtained after the third chromatography was free from the others and ultracentrifugally homogeneous.

Column chromatography on DEAE-cellulose and precipitation by ammonium sulfate

was employed for further purification of cytochromes b_4 (I) and b_4 (II). Both cytochromes b_4 (I) and b_4 (II) thus obtained were homogeneous ultracentrifugally and electrophoretically. Fig. 1 summarizes the procedure and typical results of the chromatographic purification of the chromoproteins.

Spectroscopic Properties of Cytochromes b_4 (I) and b_4 (II)—The absorption maxima, extinction coefficients and ratios of the extinction coefficient of cytochromes b_4 (I) and b_4 (II) are summarized in Table I. The absorption maxima of these cytochromes coincide closely with each other except that cytochrome b_4 (I) has another small peak at 548 mμ in addition to the main peak at 554 mμ in the reduced form. The ratio of the extinction coefficient of α to β bands of reduced cytochrome b_4 (I) is nearly equal to that of cytochrome b_4 (II). The millimolar extinction coefficient for each

Fig. 1. Chromatographic separation and purification of cytochromes.



1) Abbreviations: A. A. buffer; ammonium acetate buffer;
A. S.; ammonium sulfate;
C. F.; centrifugation.

TABLE I

Spectroscopic Properties of Cytochromes b_4 (I) and b_4 (II)

	cytochrome b_4 (I)[1]	cytochrome b_4 (II)
Absorption peaks (mμ)		
reduced form	554, 548, 521, 418	554, 521—2, 148
oxidized form	525, 414	525, 414
Millimolar extinction coefficients (E^{mM}_{1cm})		
554 mμ	36.2 (18.1)[2]	17.7
548 mμ	30.2 (15.1)	
521 mμ	34.4 (17.2)	15.4
418 mμ	307.8 (153.9)	142.3
Pyridine hemochromogens		
550 mμ	58.2 (29.1)	26.2
520 mμ	34.4 (17.2)	15.3
Extinction ratios		
E554mμ(R)/E548mμ(R)	1.2	
E554mμ(R)/E521mμ(R)	1.05	1.15
E418mμ(R)/E554mμ(R)	8.5	8.0
E554mμ(R)/E280mμ(O)	1.01	0.95

1) Two heme groups per molecule in this compound.
2) Calculated on the basis of one heme group per molecule.
R=reduced, O=oxidized

cytochrome was calculated from their extinction values and molecular weights obtained from physical measurement and iron content. Absorption spectra of these cytochromes are shown in Figs. 2 and 3, respectively.

The spectra of cytochromes b_4 (I) and b_4 (II) did not change within the pH range of 4.0 to 11.0 and also in ionic strength of 0.1 to 2.0 using sodium chloride.

In 0.25 *M* NaOH, the extinctions of α band of cytochromes b_4 (I) and b_4 (II) greatly increased, those of β band remained unchanged and the ratio of α to β bands became nearly equal to that of mammalian cytochrome c accompanied by a shift of α band to 550 mμ, and at the same time, the minor band at 548 mμ of cytochrome b_4 (I) disappeared, although it was observed in 0.20 *M* NaOH.

The Heme Groups of Cytochromes b_4 (I) and b_4 (II)—The heme groups of these cytochromes did not split from the proteins by treatment with 0.1 *M* HCl-methylethylketone nor by usual method of HCl-acetone. Furthermore, these cytochromes exhibited typical c-type pyridine hemochromogen spectra with maxima at 550, 520 and 415 mμ. The heme content of both cytochromes b_4 (I) and b_4 (II) was determined from the spectra of pyridine hemochromogens and molecular weights were calculated from physical measurements. The results indicated that cytochrome b_4 (I) might contain 2 heme groups per molecule and cytochrome b_4 (II), 1 heme group.

Physicochemical Properties of Cytochromes b_4 (I) and b_4 (II)—A mumber of physicochemical properties of these cytochromes are summarized in Table II.

Sedimentation velocities were measured at protein concentrations of 1.54, 0.66, 0.44 and 0.22 per cent for cytochrome b_4 (I) and 0.29 per cent for cytochrome b_4 (II) and were found to be independent of protein concentration. The minimal molecular weights calculated from the iron content were 9,300 for cytochrome b_4 (I) and 14,800 for cytochrome b_4 (II). On the other hand, the molecular weight of cytochrome b_4 (I) calculated from S, D values and assuming as V=0.71 was

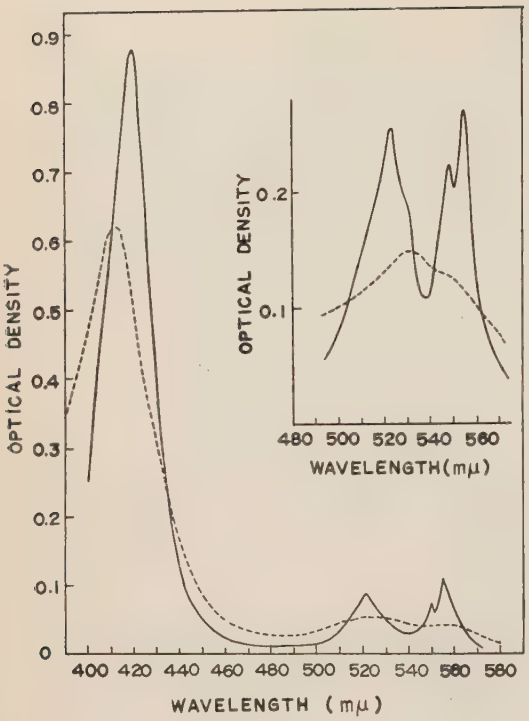


Fig. 2. Absorption spectrum of cytochrome b_4(I).

Both cytochromes b_4 (I) and b_4 (II) were reduced by the addition of a few mg. of sodium hydrosulfite. ——: Reduced form. .....: Oxidized form.

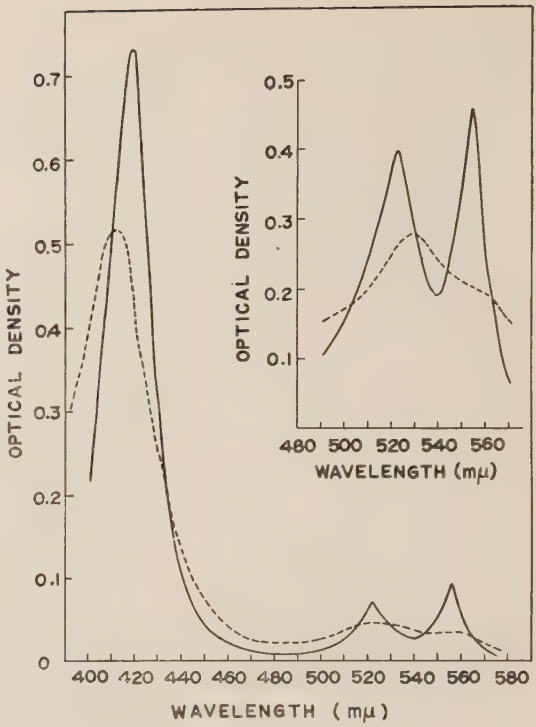


Fig. 3. Absorption spectrum of cytochrome b_4 (II).

Table II

Physicochemical Properties of Cytochromes b_4(I) and b_4 (II)

	cytochrome b_4 (I)	cytochrome b_4 (II)
$s_{20,w}$	2.20S[1]	2.17S[1]
$D_{20,w}$	10.2×10^{-7}cm^{2}sec^{-1}	——
f/f_0	1.12	——
Minimum molecular weight	9,300[2]	14,800[2]
Molecular Weight	18,000[3]	about 18,000[3]
Electrophoretic mobility (U)	-9.6×10^{-5}cm^2.sec^{-1}volt.$^{-1}$	-12.7×10^{-5}cm^2.sec^{-1}volt.$^{-1}$
p^I	3.2	near 3
E_0' (pH 7)	+0.113 volt.	+0.18 volt.
Iron content (%)	0.61	0.38

1) Experimental conditions: see the text.
2) Calculated from iron content.
3) Calculated from the values obtained by physical measurements.

18,000 and that of cytochrome b_4 (II) calculated from S value and assuming as D and V values being nearly equal to the figures for cytochrome b_4 (I) was about 18,000.

Electrophoretic mobilities for these cytochromes were found to be -9.6×10^{-5} cm.^{2} sec^{-1} volt.$^{-1}$ for cytochrome b_4 (I) and -12.7×10^{-5} cm.^{2} sec^{-1} volt.$^{-1}$ for cytochrome b_4 (II) at pH 7.0.

The effect of pH on the electrophoretic mobility of cytochrome b_4 (I) is shown in Fig. 4. Isoelectric point of this cytochrome determined by extrapolation of mobility values was pH 3.2.

Oxidation-reduction potentials were measured using ferri-ferro oxalate system at pH 7.0 and 25°C. As shown in Table II, cytochrome b_4 (I) had somewhat low potential (+0.113 volt.) as compared to that of cytochrome b_4 (II), the potential of the latter was +0.180 volt. The influence of pH upon oxidation-reduction potential was studied on cytochrome b_4 (I) only and the result is illustrated in Fig. 5. The potential was

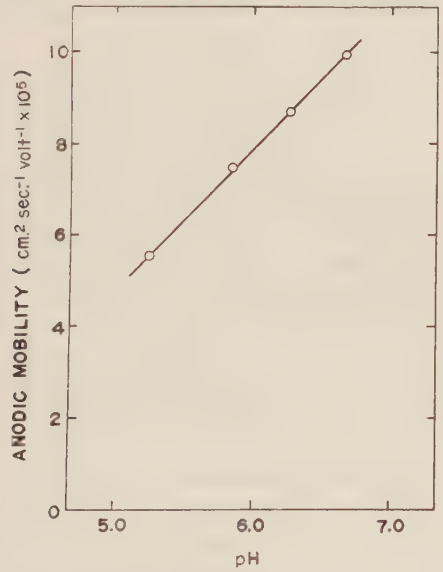


Fig. 4. The effect of pH on the electrophoretic mobility of cytochrome b_4 (I).

Experimental conditions: Each aliquot of the cytochrome (3.4 mg.) were dialyzed against phosphate buffer which was used at all pHs. Electrophoresis was carried out at 2.4°C, 6.5 mA and 100 to 125 volt.

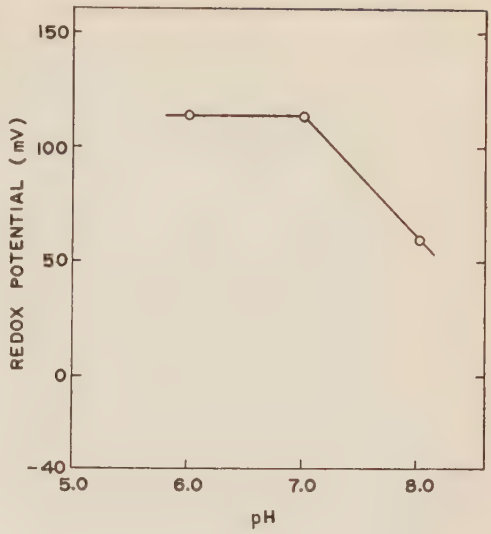


Fig. 5. The effect of pH on oxidation-reduction potential of cytochrome b_4 (I).

Experimental conditions: Reaction mixture contained Na-oxalate 1.5 m moles, ferric ammonium sulfate 6 μmoles, cyt. b_4 (I) 3 μmoles, phosphate buffer 0.68 mmole in open cuvette (3 ml.). In control cuvette cytochrome was omitted from the complete reaction mixture. Ferrous ammonium sulfate ($2.5\times10^{-3}M$) was gradually added into the both cuvettes and the changes of extinction of the cytochrome at 418 mμ was measured.

maintained at +0.113 volt. between pH 6.0 and 7.0 under the conditions employed, but above pH 7.0 it changed by −0.06 volt. per pH unit and gave +0.059 volt. at pH 8.0.

Absolute Amounts of Cytochromes b_4 (I), b_4 (II) and c^{551}—Five soluble chromoproteins have been obtained from the *Micrococcus* grown both aerobically and anaerobically in the presence of nitrate. Absolute amounts of cytochromes b_4(I), b_4(II) and c^{551} are summarized in Table III.

Enzymatic Properties of Cytochromes b_4 (I) and b_4 (II)—Reduction of cytochrome b_4 (I) proceeded faster than that of cytochrome b_4 (II) by succinate, DPNH and formate in the presence of the sonicate of this *Micrococcus* under anerobic condition and both cytochromes b_4 (I) and b_4 (II) were reoxidized to nearly the same extent by the addition of nitrate, nitrite and hydroxylamine. Oxygen

Table III

Amounts of Cytochromes b_4 (I), b_4 (II) and c^{551} Obtained from Aerobic and Anaerobic Cellt Grown with Nitrate

Condition of cultivation	cytochrome b_4 (I) (mg.)	cytochrome b_4 (II) (mg.)	cytochrome c^{551} (mg.)
Aerobic	43.2[1)]	26.7	54.6
Anaerobic	158	3.4	24

1) These figures indicate cytochrome content (mg.) per 100 g. of dry cell.

could also reoxidize these cytochromes but in a slower rate. The results of reduction of cytochromes b_4 (I) and b_4 (II) by succinic-cytochrome reductase system and reoxidation of them by cytochrome oxidase system were shown in Fig. 6 (a) and (b).

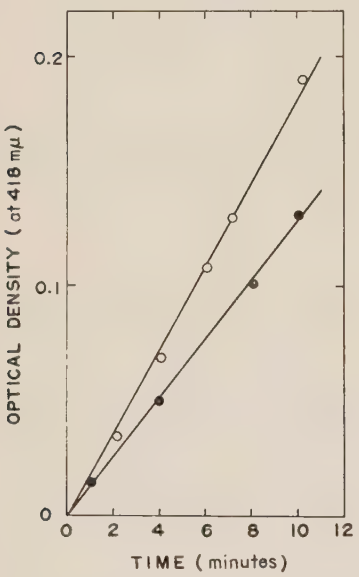


Fig. 6 (a). Reduction of cytochromes b_4 (I) and b_4 (II) by the *Micrococcus* succinic-cytochrome reductase. (○) Succinic-cytochrome b_4 (I) reductase. (●) Succinic-cytochrome b_4 (II) reductase. Reaction mixture contained 0.5 *M* phosphate buffer (pH 6.8) 0.3 ml., 0.1 *M* potassium cyanide 0.3 ml., 0.2 *M* sodium succinate 0.2 ml., cytochrome 3 mg. enzyme solu- tion 0.2 ml. (8.2 mg. protein) and distilled water in total volume of 3.0 ml. Reactions were started by addition of succinate.

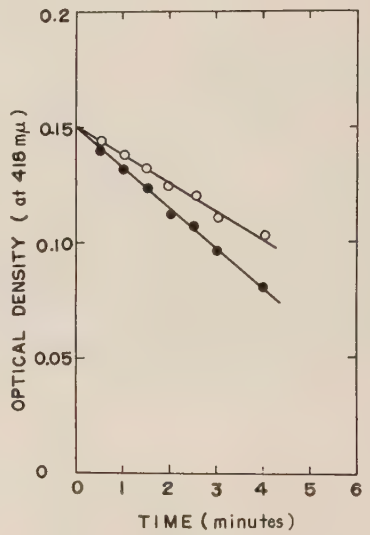


Fig. 6 (b). Reoxidation of cytochromes b_4 (I) and b_4 (II) by the *Micrococcus* cytochrome oxidase. (○) Cytochrome b_4 (I) oxidase. (●) Cytochrome b_4 (II) oxidase.

Reaction mixture; each cuvette contained 0.5 *M* phosphate buffer (pH 6.8) 0.3 ml., 5 *M* sodium chloride 0.4 ml., enzyme solution 0.5 ml. (15 mg. protein), reduced cytochrome (2 mg.) and distilled water in total volume of 3.0 ml. Reactions were started by addition of cytochrome.

DISCUSSION

All of the five chromoproteins isolated from this *Micrococcus* had markedly acidic nature, so a chromatographic separation on DEAE-cellulose was very useful for the purification of these chromoproteins. Cytochromes b_4 (I) and b_4 (II) showed unusual properties as compared to those of cytochromes

of type-c found in other sources. They differed from those of other c-type cytochromes, especially in their absorption spectra, the ratios of α to β bands and of Soret to α bands.

Furthermore, regardless of its purity, reduced cytochrome b_4 (I) exhibited an absorption spectrum with double α bands even at room temperature.

By the titration with ferri-ferro oxalate buffer, cytochrome b_4 (I) was reduced and the ratio of reduced to oxidized forms were substantially equivalent at all of the wavelengths investigated, namely, at 554, 548 and 418 mμ. From these facts, the double α bands observed appeared to be attributed to specific properties of a single entity, *i.e.* cytochrome b_4 (I).

Davenport and Hill (*19*), using low dispersion spectroscope, showed that reduced cytochrome f exhibited a spectrum with double α bands at 556 and 551 mμ at pH 10.8. A more distinct splitting of α band of cytochrome f has been shown by Bonner (*20*) in the absorption spectrum observed at -190°C.

Recently, Yamanaka *et al.* (*21*) and Horio *et al.* (*22*) have reported on cytochromes having double α bands in reduced forms at room temperature.

Cytochromes b_4 (I) and b_4 (II) exhibited typical c-type pyridine hemochromogen spectra with maxima at 550, 520 and 415 mμ. Moreover, their heme groups did not split from the proteins by treatment with HCl-methylketone. The heme groups of these cytochromes therefore appear to be similar to that of mammalian cytochrome c.

Oxidation-reduction potentials of these cytochromes are lower than that of mammalian cytochrome c. As is shown in Fig. 4, the potential of cytochrome b_4 (I) varied by -0.06 volt. with a change of 1 pH unit at above pH 7; this fact seems to indicate that ferricytochrome b_4 (I) has a certain group being dissociable at pHs higher than 7.

Enzymatic activity of cytochrome b_4 (I) as an intermediate carrier in an electron transfer system from DPNH and succinate to nitrite and hydroxylamine was confirmed in the crude extracts of this *Micrococcus* (*11*, *12*). Purified cytochromes b_4 (I) and b_4 (II) were also shown to function as intermediate electron carriers in a formate-nitrate system as well as in the systems mentioned above. Difference in the function of cytochromes b_4 (I) and b_4 (II) in these systems is not clear at the moment. Reduced cytochromes c^{551}, b_4 (I) and b_4 (II) and brown protein were reoxidized by oxygen in the presence of subcellular particle of this *Micrococcus*. In the case of this *Micrococcus*-cytochrome oxidase system, cytochrome c^{551} appeared to be the best electron donor among them. Relative amount of cytochromes b_4 (I), b_4 (II) and c^{551} obtained from the cells grown aerobically and anaerobically in the presence of nitrate was shown in Table III. Under anaerobic condition, total cytochrome content increased markedly, cytochrome b_4 (I) especially being highly dominant.

Similar phenomena have been observed by many workers in a number of different organisms (*1*, *23*, *24*). Under the same condition, an increase in the activities of NaR, NiR, HdR and *p*-phenylenediamine oxidase was also observed (*13*).

In addition to cytochromes of c-type and HdR (cytochrome 625, 553), most recently, a cytochrome of type-b having absorption maxima at 560, 530 and 428 mμ in reduced form was isolated from this *Micrococcus* grown anaerobically. Its prosthetic group was easily splittable from the protein with HCl-acetone and a reduced pyridine hamochromogen derived from it exhibited a spectrum of pyridine protohemochromogen with maxima at 557, 525 and 420 mμ. An attempt to isolate this cytochrome from the cells grown aerobically have not yet been done, though a more distinct band near at 560 mμ which seemed to be due to cytochrome of type-b was observed by a hand spectroscope.

From the results as mentioned above, it may well be assumed that in the aerobic cells cytochrome 560, cytochromes b_4 (I), b_4 (II) and c^{551} may participate in oxygen respiration as intermediary electron carriers in the order shown above (pathway 1) and that in the

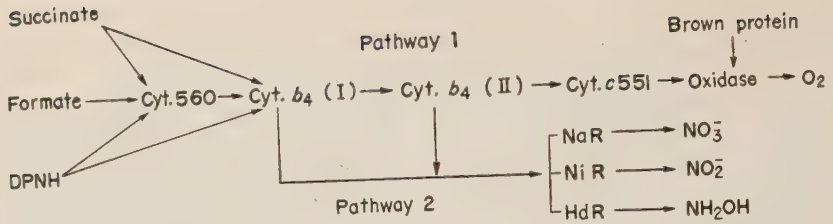


Fig. 7. A possible scheme of electron transfer sequence from succinate, DPNH and formate to oxygen, nitrate, nitrite and hydroxylamine.

anaerobic cells pathway 1 may decline and an alternative shunt in which cytochrome 560 and cytochrome b_4 (I) participate may come into operation (pathway 2).

A postulated formulation for the electron transfer sequence participating in nitrate and oxygen respirations is as follows (Fig. 7). More detailed investigations on the interrelationship of cytochromes b_4 (I) and b_4 (II) and also of other chromoproteins are under progress. Properties of cytochrome c^{551}, brown protein and cytochrome type-b will be given elsewhere.

SUMMARY

1. Five chromoproteins: cytochrome c^{551}, brown protein, cytochromes b_4 (I), b_4 (II) and HdR (cyt. 625, 553) were obtained in soluble form from a halotolerant *Micrococcus* and were purified by chromatography on DEAE-cellulose column.

2. Reduced cytochrome b_4 (I) exhibited an absorption spectrum with double α bands at 554 and 548 mμ while reduced cytochrome b_4 (II) had single α band at 554–555 mμ.

3. Cytochrome b_4 (I) had two heme groups per molecule, on the other hand, cytochrome b_4 (II) had one heme group per molecule.

4. Oxidation-reduction potential for cytochromes b_4 (I) and b_4 (II) were +0.113 volt. and 0.180 volt., respectively.

5. These cytochromes exhibited c-type pyridine hemochromogen spectra and their heme groups did not split from the proteins by treatment with HCl-acetone and also HCl-methylethylketone.

6. They were reduced by DPNH, succinate and formate and were rapidly reoxidized by the addition of nitrate, nitrite and hydroxylamine in the presence of crude extracts of the *Micrococcus*. Oxygen could also reoxidize them but at a slower rate.

7. A possible scheme of electron transfer from DPNH, succinate and formate via cytochrome of type-b, cytochromes b_4 (I), b_4 (II) and c^{551} to nitrate, nitrate, hydroxylamine and oxygen was presented.

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A New Azomercurial for the Quantitative Determination of Sulfhydryl Groups of Mercaptans and Proteins

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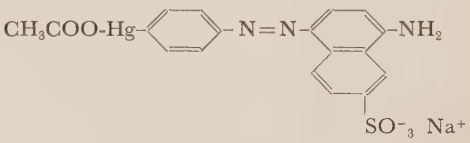
Many procedures for the determination of sulfhydryl groups of mercaptans, proteins and enzymes have been devised, because of the playing their important roles in the biological kingdom (*1*, *2*). Among them, the techniques of using organic mercurials have been extensively developed and, in particular, the spectrophotometric procedure of Boyer (*3*) based on the spectral change of *p*-chloromercuribenzoate (PCMB) with sulfhydryl groups at 250–255 mμ has been widely applied, because of its high sensitivity and rapidity in measurements. However, as Boyer pointed out, this procedure when applied for proteins has the disadvantage that the measurements have to be made in the spectral region of strong light absorption by proteins. The measurements of various biological extracts and intact materials are difficult to be conducted, since they usually contain, beside proteins and peptides, nucleic acids and their bases which absorb light more strongly than proteins do in the spectral region of PCMB. Many attempts to find mercurials that would give an appreciable spectral change in the visible region have so far resulted to the failure (*3*, *4*). A colored mercurial, 1-(4-chloromercuriphenylazo)-naphthol-2 synthesized by Bennett (*5*, *6*) is known as a satisfactory cytochemical reagent to observe the distribution of sulfhydryl groups in cells under a microscope, but for quantitative measurements, the excess of remaining non-reacted dye has to be separated by the cumbersome distribution procedure, because it gives the small spectral change on the reaction.

The present paper deals with a newly synthesized organic mercurial, which dissolves fairly well in buffers and gives considerable spectral changes in the visible region on reacting with sulfhydryl groups. This compound is sodium 4-(4-acetoxymercuriphenylazo)-1, 7-Cleve's acid (1-naphthylamine-7-sulfonic acid) having the structure shown below, and is abbreviated to MPAC in this paper. A high specificity of this compound to sulfhydryl groups was obtained when employed with glycine, which was found to suppress the reactions of MPAC with various ions or groups other than sulfhydryl groups. The properties and application of this azomercurial for the determination of sulfydryl groups of organic mercaptans as well as proteins are described herewith.



Structure of MPAC

EXPERIMENTAL

Preparation of MPAC—3.5 g. of *p*-aminophenylmercuric acetate, which was prepared from aniline and mercuric acetate according to the method Dimroth (*7*), was diazotized with 0.7 g. of sodium nitrite in 50 ml. of 50% acetic acid at −5°C (*5*, *8*). The filtered diazo solution was coupled slowly with 2.5 g. of 1, 7-Cleve's acid in 1 liter of ice water saturated with sodium acetate. After standing overnight, the precipitates were collected by filtration, dissolved in water, filtered and salted out with sodium acetate. The precipitates were washed with 90% ethanol several times to remove other colored by-products and sodium acetate. The dye thus obtained was dried over sulfuric acid *in vaccuo* at room temperature. Yield, 0.4 g.

Anal.; Calcd. for $C_{18}H_{14}O_5N_3HgSNa$, N 6.91%, found N 6.74%. This sample gave one spot on development of one-dimensional paper chromatography with the mixture of butanol, pyridine, water and conc. aq. ammonia (4:5:4:1) or phenol saturated with water as solvent.

Preparation of Proteins—The sample egg albumin was prepared by the method of Kekwick and Cannan (*9*), recrystallized twice and dialysed against water until being free from sulfate. The concentrations were determined gravimetrically, assuming 46,000 as the molecular weight (*10*). The bovine serum albumin was a commercial crystalline product of Behringwerk and its concentration was calculated from the absorbance value at 280 mμ, with aid of the molecular weight of 70,000 and $E^{1\%}_{1cm.}=6.6$ reported by Simpson, Li and Livans (*11*). The crystalline bovine γ-globulin was purchased from Tokyo Kasei Co. The sample α-casein was prepared by Dr. T. Nagumo following the procedure of Warner from skim milk (*12*) and the molecular weight was assumed to be 121,800 (*12*) in the gravimetric determination of its concentration.

Spectroscopic Measurements—The well dried MPAC was first dissolved in 0.02 *M* NaOH solution, and then diluted with a buffer of a required pH. 0.1 *M* glycine-0.04 *M* acetate was mostly used for the buffer. A slight deviation of pH from a desired value was, then, adjusted with a small amount of acetic acid or a concentrated NaOH aq. The buffered dye solution showed no deterioration for two or three days when stored in a refrigerator. 5.0 ml. of MPAC solution in the glycine-acetate buffer was added to 0.5 ml. of a protein or mercaptan solution in the same buffer at 28°C, and the absorption spectrum of this mixture was observed with a Hitachi spectrophotometer, model EPU-2A, using 1 cm. cells. In most of the observations, the difference spectrum in terms of ΔE was directly observed.

RESULTS AND DISCUSSION

Specificity of MPAC without Glycine—Curve A in Fig. 1 is the absorption spectrum of MPAC in 0.1 *M* acetate of pH 8.0. The spectrum shows a flat band at 450 mμ, and the molar extinction coefficient (ε) at the maximum position was calculated to be 1.73×10^4 cm.$^{-1}M^{-1}$. The change of the solvent from acetate to 0.05 *M* phosphate buffer of pH 8.0 caused no variation of the spectrum. Upon addition of excess cysteine to this dye solution the band was considerably enhanced (curve B in Fig. 1). The increase of molar extinction coefficient ($\Delta\varepsilon$) in this transformation of band with excess cysteine was 4.0×10^3 at 450 mμ. The addition of egg albumin also raised the band higher ($\Delta\varepsilon$ at 452 mμ = 1.02×10^4) and caused a slight shift of band to 451 mμ accompanied by an appreciable drop of absorbance below 410 mμ (curve C in Fig. 1). Curves A and B in Fig. 2 are the difference spectra between these transformations of band, which were observed of MPAC and its mixture with cysteine and egg albumin, respectively. The ΔE values thus obtained are large enough to apply them for the determination of the sulfhydryl group, if the dye shows no spectral change with substances containing no sulfhydryl group. However, the similar tests for the mixtures of MPAC with some amino acids other than cysteine, anions such as halides, and bovine

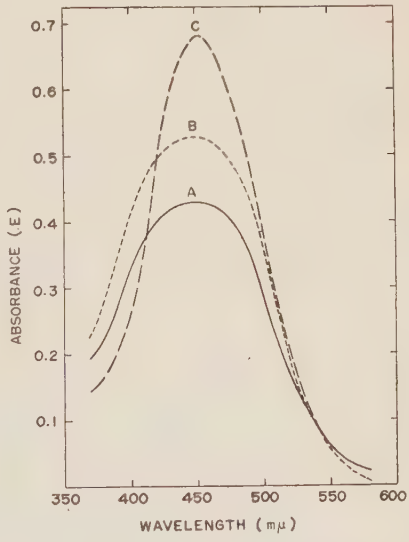


FIG. 1. Absorption spectra of 2.5×10^{-5} *M* MPAC in 0.1 *M* acetate buffer of pH 8.0 (curve A) and its mixtures with 5.0×10^{-5} *M* cysteine (curve B) and 5.0×10^{-5} *M* egg albumin (curve C).

γ-globulin with no sulfhydryl group showed spectral changes being comparable in order with those observed with the sulfhydryl groups containing substances, and precipitates were formed in some cases. Examples are shown by curves C and D in Fig. 2, which are the difference spectra obtained for

mixtures of MPAC with cystine and bovine γ-globulin, respectively. These non-specific combinations of MPAC seem to be the same phenomena as observed by Barron (*13*) who concluded that mercuric chloride, phenyl mercuric nitrate and certain organic mercurials might cambine with carboxyl or amino groups of proteins.

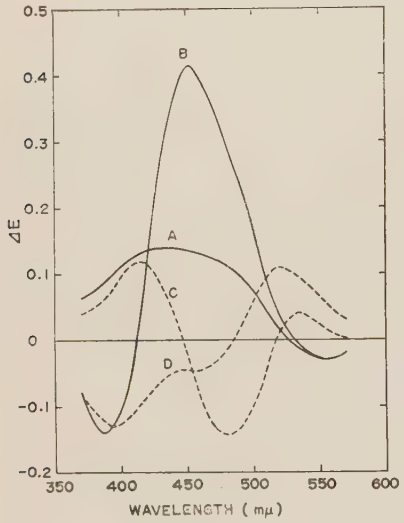


Fig. 2. Difference spectra between 4.25×10^{-5} *M* MPAC in 0.05 *M* phosphate buffer of pH 8.0 and its mixtures with 1.0×10^{-4} *M* cysteine (curve A), 5.0×10^{-5} *M* egg albumin (curve B), 8.0×10^{-5} *M* cystine (curve C) and 0.1% bovine γ-globulin (curve D).

Specificity of MPAC in Glycine-Acetate Buffer —Two different mechanisms may be proposed as to the non-specific reactions of MPAC. Gurd and Wilcox (*14*) pointed out that anionic groups combining with the mercury atom of mercurials may ionize in water, resulting in non-specific combination of the atom with carboxyl or amino groups of proteins. The acetoxy group of MPAC may also ionize at least partially, and R-Hg^+ residue thus formed may combine with anions, amino acids or various residues of proteins. Another mechanism is the combination of amino or sulfonic group of the MPAC molecule with ions or ionic sites of proteins. This type of combination which was studied by Klotz (*15*), is discussed later and is proved to be negligible under the experimental conditions adopted in the present study.

According to the former mechanism of reaction, the undesirable combination of MPAC occurs owing to the ionizable character of the mercury atom. One may, therefore, improve the specificity of MPAC by replacement of the acetoxy group by an anion or residue having a higher affinity or just by mixing MPAC in solution with a compound capable of forming such an anion or residue. Attempts to synthesize mercurials having such residues as halides in place of acetoxy group, however, were unsuccessful. According to the observation made by Klotz (*4*), the coexistence of glycine greatly increases the solubility and the specificity of 4-(*p*-dimethylaminobenzenazo)-phenylmercuric acetate. This phenomenon was interpreted as being due to the complex formation between the dye and glycine, which may be expressed by the following scheme;

$$\text{R-Hg-OOCCH}_3+\text{H}_2\text{NCH}_2\text{COO}^- \longrightarrow \text{R-Hg}^+\text{-NH}_2\text{CH}_2\text{COO}^-+\text{CH}_3\text{COO}^- \qquad \text{(I)}$$

The coexistence of glycine was also found to be effective to eliminate the undesirable combination of MPAC.

Several preliminary experiments were made for the mixture of MPAC and glycine, before examining the specificity. The spectrum of MPAC in 0.1 *M* glycine-0.04 *M* acetate buffer of pH 8.0 showed an absorption band at 450 mμ (curve A in Fig. 3), the same position as that observed without glycine. However, the ε value at the maximum is 1.94×10^4, being higher than the value obtained without glycine, and this is considered to be an indication that the exchange reaction shown by equation (I) occurs in the glycine-acetate buffer. The increase of glycine concentration more than 0.1 *M* did not change the band height. This fact indicates that the reaction proceeds to completion with 0.1 *M* glycine. The spectrum shown by curve A in Fig. 3 and the ε value written above are those of the MPAC-glycine complex. The change of pH between 7 and 10 showed practically no variation of

the spectrum as indicated by the absorbance value at 450 mμ shown by curve A in Fig. 4. The slight lowering of absorbance observed above pH 10 may arise from replacement of

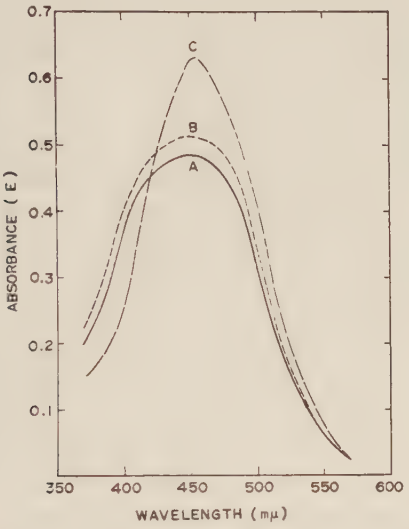


FIG. 3. Absorption spectra of 5.0×10^{-5} *M* MPAC in 0.1 *M* glycine-0.04 *M* acetate buffer of pH 8.0 (curve A) and its mixtures with 6.0×10^{-5} *M* cysteine (curve B) and 3.0×10^{-5} *M* egg albumin (curve C).

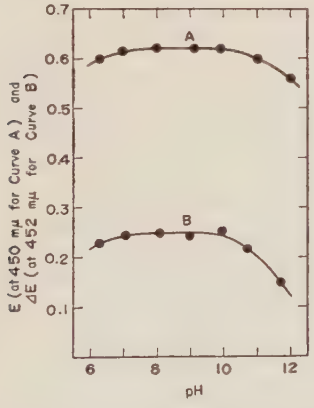


FIG. 4. The pH dependencies of absorbance value at 450 mμ of 3.2×10^{-5} *M* MPAC in the glycine-acetate buffer (curve A) and the ΔE value for the mixture of 4.37×10^{-5} *M* MPAC and 1.95×10^{-5} *M* egg albumin in the same buffer (curve B).

the glycine residue by hydroxy ion (*2*). In Fig. 5 is shown the proportionality between the absorbance value at 450 mμ and concentration in the glycine-acetate buffer of pH 8.0. The solution obeys Beer's law up to the absorbance value of 2.0, which is the limit of observation with commonly used spectrophotometers.

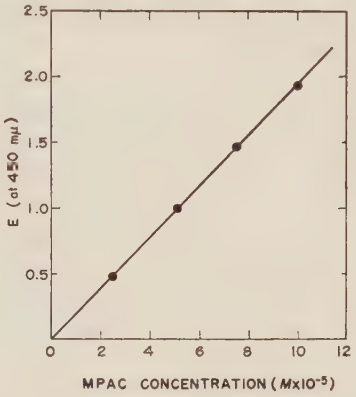


FIG. 5. Relation between MPAC concentration and the absorbance value at 450 mμ in the glycine-acetate buffer of pH 8.0.

MPAC is very soluble in glycine-acetate buffers above pH 7.0. At pH 6.0, precipitates were formed on standing, and precipitation started immediately after the pH of the solution was lowered below 5.6. The solubility in the buffer of pH 8.0 was estimated to the order of 10^{-2} *M*, being too high to determine it more exactly. The solubility of 4-(*p*-dimethylaminobenzenazo)-phenylmercuric acetate observed by Klotz (*4*) is as low as 6×10^{-6} *M* even in glycine-acetate buffer of pH 9.6, and 1-(4-chloromercuriphenylazo)-naphtol-2 synthesized by Bennett (*5*, *6*) is practically insoluble in water, so that this compound has to be used with organic solvents. The high solubility of MPAC, which may be mostly due to the sulfonic group of the molecule, enables us to adjust its concentration easily and to obtain an appropriate reading of ΔE.

Using 4.0×10^{-5} *M* MPAC in the glycine-acetate buffer of pH 8.0, the effect of substances containing no sulfhydryl group was examined. The addition of 1.0×10^{-2} *M* arginine, glutamic acid, histidine and methionine, 5×10^{-4} *M* cystine, 2×10^{-3} *M* tyrosine, 0.1 *M* citric acid and casein hydrolysate in 0.1% nitrogen

content caused no appreciable spectral change. The following inorganic ions tested had also no effect on the spectrum; *i.e.* 0.1 *M* phosphate, sulfate, chloride, carbonate and nitrate; and 1.0×10^{-3} *M* Zn^{++}, Ca^{++}, Cu^{++} and Mg^{++}. On the other hand, the addition of 1.0×10^{-3} *M* Fe^{+++} altered the spectrum considerably and increased the band height. This effect could, however, be eliminated completely, when 1.0×10^{-3} *M* EDTA (ethylenediaminetetraacetic acid) was added simultaneously. The addition of the same concentration of EDTA without ferric ion as a control test showed no effect on the spectrum. In order to see the effect of proteins having no sulfhydryl group, 0.1% bovine γ-globulin and 1.0×10^{-5} *M* α-casein were mixed with 4.3×10^{-5} *M* MPAC at pH 8.0, respectively. The result showed no appreciable change of the spectrum.

Trials to improve the specificity of MPAC by mixing it with inorganic anions including halides, sulfate, and various buffers in place of glycine were unsuccessful. Glycine is the best reagent to be mixed with MPAC, since it acts both as a buffer with acetate and as a suppressor of the reaction of MPAC with ions or substances with no sulfhydryl group.

Spectral Changes by Reaction of the MPAC-Glycine Complex with Mercaptans—When various mercaptans (cysteine, glutathione, mercaptoethanol and thioglycolic acid) were added to MPAC solution in the glycine-acetate buffer of pH 8.0, the reaction was completed within approximately 10 minutes at 28°C. Curve B in Fig. 3 shows an example of the resultant spectrum of the mixture of MPAC and cysteine. The maximum increase of absorbance caused by mercaptide formation was observed at 470 mμ with excess cysteine was 1.39×10^{3}. The spectra obtained with excess mercaptoethanol, thioglycolic acid and glutathione were the same in shape and position as curve B in Fig. 3. The $\Delta\varepsilon$ values at 470 mμ obtained for these compounds were 1.38×10^{3}, 1.36×10^{3} and 1.40×10^{3}, respectively, being in good agreement with the value obtained with cysteine.

As shown by curve A in Fig. 6, the ΔE value increases proportionally with increase of glutathione concentration, and reached a constant value. When MPAC concentration was increased to twice the value used for the observation of curve A, the height of the constant level of ΔE was doubled (curve B in Fig. 6). From the intersection of the two straight lines in the figure, the amount of glutathione consumed for its combination with all the dye dissolved was estimated for each curve. The moles of MPAC combined with one mole of glutathione was 1.06 from curve A and 0.95 from curve B. It was thus established that the number of sulfhydryl groups of mercaptan molecules can be determined within several percent errors.

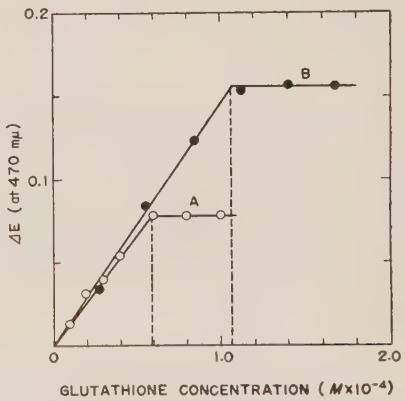


Fig. 6. Relation between ΔE value at 470 mμ and glutathione concentration in the glycine-acetate buffer of pH 8.0. The concentration of MPAC was fixed at 5.6×10^{-5} *M* for curve A and 1.12×10^{4} *M* for curve B.

The dye seems to be applicable for the chromatographic sepapation of mercaptans. A preliminary experiments for a mixture of MPAC, cysteine and glutathione showed three spots on paper chromatography, which were identified as those of free dye and its complexes with these mercaptans. The studies of biological samples and their extracts with this dye are contemplated and left open to the further investigations.

Spectral Changes by Reaction of the MPAC-Glycine Complex with Proteins—Egg albumin and bovine serum albumin were chosen as the representative samples of proteins containing sulfhydryl groups, and bovine γ-globulin and

α-casein as proteins with no sulfhydryl group, and their reactions with the MPAC-glycine complex were scrutinized under various conditions. Curve C in Fig. 3 is the spectrum of the mixture of MPAC with egg albumin in the glycine-acetate buffer of pH 8.0. The red shift brought about by their combination is small, being 1 mμ or less, but the band was sharpened and intensified considerably. The difference spectrum observed with excess egg albumin showed a positive band at 452 mμ and a negative band at 390 mμ.

The time course of the reaction, following the absorbance change at 452 mμ, is shown by curve in Fig. 7, where the ΔE value reaches a constant level within one hour. Measurements with various concentrations of MPAC and egg albumin revealed that two hours are sufficient for the completion of the spectral changes.

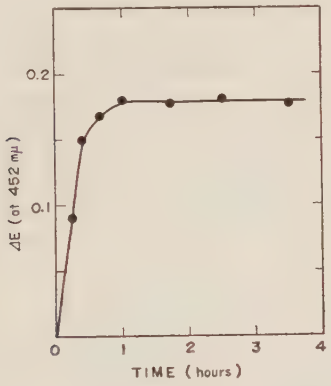


Fig. 7. Time course of absorbance change at 452 mμ in the reaction between 3.19×10^{-5} *M* MPAC and 1.5×10^{-5} *M* egg albumin in the glycine-acetate buffer of pH 8.0.

The value of Δε at 450 mμ observed with excess egg albumin was 5.77×10^{3}, being comparable to the Δε value (6.3×10^{3}) of PCMB (*3*) at 255 mμ and pH 4.6. The pH dependency of the absorbance change at 452 mμ was measured with MPAC and egg albumin, and the result is shown by curve B in Fig. 4, which indicates no variation of ΔE between pH 7 and 10, but a remarkable drop above pH 10.

The relation obtained between the ΔE value at 452 mμ and egg albumin concentration is shown in Fig. 8, which was observed with 4.37×10^{-5} *M* MPAC in the glycine-acetate buffer of pH 8.0. The similar observations at other pH's between 8 and 10 showed practically the same curve as that in Fig. 8. The amount of egg albumin reacted with MPAC was estimated by extrapolation of the two straight lines in the figure. The result showed 3.1 moles of MPAC combined with one mole egg albumin. Boyer (*3*), by use of PCMB, obtained 3.2 as the number of the sulfhydryl groups, and MacDonnell, Silva and Feenay (*15*) obtained 2.9 at pH 5.3 by their titration method with PCMB. These values agree with the value obtained by use of MPAC.

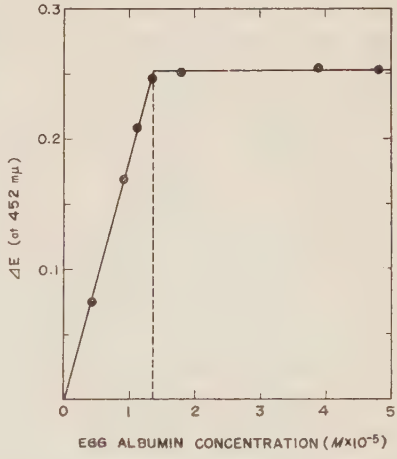


Fig. 8. Relation between ΔE value at 452 mμ and egg albumin concentration in the glycine-acetate buffer of pH 8.0. The concentration of MPAC was fixed at 4.37×10^{-5} *M*.

Based upon the same principle as above, the number of sulfhydryl groups in the bovine serum albumin molecule was determined at pH 8.0; that was 0.74. Bennett (*6*) reported that the number of sulfhydryl groups in the units per 100,000 g. of bovine serum albumin was 1.01 by using mercuric iodide, 1.00 by silver nitrate amperometric titration method and 1.02 by using Bennett's mercury orange. The above value obtained with MPAC corresponds to 1.05 in the unit used by Bennett, and agrees well with these values

obtained by various techniques.

Effect of Non-Sulfhydryl Containing Substances on the Reaction—The effect of the second component with no sulfhydryl group on the reaction between $5.8\times10^{-6}\,M$ egg albumin and $2.1\times10^{-5}\,M$ MPAC in the glycine-acetate buffer of pH 8.0 was examined. Examination with 0.1 M phosphate, acetate, chloride, carbonate, nitrate and citrate as the component showed no effect on the ΔE value at 452 mμ obtained by the reaction of MPAC with egg albumin. The similar experiments with $1.0\times10^{-3}\,M$ of Zn^{++}, Ca^{++}, Cu^{++} and Mg^{++} did not also inhibit the reaction. The addition of $1.0\times10^{-3}\,M$ Fe^{+++} increased the ΔE value remarkably. The increased ΔE value was, however, brought back to the original value when $1.0\times10^{-3}\,M$ EDTA was added simultaneously or later. A blank test with the same concentration of EDTA without ferric ion showed no effect on the ΔE value. However, caution has to be paid in the use of EDTA, since the increase of EDTA concentration to $5\times10^{-3}\,M$ caused 20% reduction of the ΔE value. Mercuric ion and PCMB have a strong effect on the reaction. The addition of $2.5\times10^{-5}\,M$ mercuric chloride to $7.7\times10^{-6}\,M$ egg albumin or the addition of $1.0\times10^{-4}\,M$ PCMB to $3.2\times10^{-5}\,M$ egg albumin completely inhibited the reaction with $4.8\times10^{-5}\,M$ MPAC. The same amino acids as tested previously and bovine γ-globulin and α-casein showed no effect on the reaction between egg albumin and MPAC in the glycine-acetate buffer. These experimental results support the point of view that spectral changes observed are caused by the replacement of glycine residue of the complex by sulfhydryl group of mercaptans or proteins, and eliminates another possible mechanism, the reaction of the sulfonic or amino group of MPAC with ions or ionic sites of proteins.

SUMMARY

A new azomercurial, sodium 4-(4-acetoxymercuriphenylzao)-1, 7-Cleve's acid (abbreviated to MPAC) was synthesized, and its applicability for the spectrophotometric and quantitative determination of sulfhydryl groups of mercaptans and proteins was studied under various experimental conditions. The dye, when used with glycine in solution, has a high specificity of reacting only with sulfhydryl groups, and its application to mercaptans and proteins gave us the same contents of sulfhydryl groups as observed by other techniques. The highly specific reaction of MPAC in the glycine-acetate buffer was due to the formation of a MPAC-glycine complex, in which the glycine residue having an appropriate affinity with the mercury atom suppressed the non-specific reactions with ions or radicals having a weaker affinity with the mercury atom. The dye in its application has two major advantages; i) we observe the spectral change in the visible region, where most proteins and extracts from biological materials absorb light weakly and ii) the solubility of the dye in water or buffers is much higher than those of the mercurials so far synthesized, so that we may obtain a greater reading of absorbance change brought about by mercaptide formation.

The author wishes to express his gratitude to Prof. K. Shibata for his valuable advices and encouragement throughout this investigation.

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Studies on the Protein Synthesis in Silkglands

I. Transfer of Radioactivity from Prelabelled Cell Debris to Particulate Fractions in the Cell Free Systems of the Posterior Silkgland*

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Silk fibroin, one of the constituents of silk protein is synthesized in the silkworm's posterior silkgland. Silk fibroin contains glycine in the amount of 44 per cent. The glycine which is administered to the silkwom is almost exclusively incorporated into the fibroin (approximately by 95 per cent) (*1*, *2*), so that it is quite advantageous to investigate the biosynthesis of fibrous protein using silkworms as the experimental materials.

When silkgland preparations are homogenized and cell structures are destroyed, however, the ability to incorporate C^{14}-amino acid into protein is almost completely lost (*3*), consequently a marked protein synthesis can not occur in the homogenates. These facts impressed us as to the possible involvement of "cell structures" or "membranous structures" in the biosynthesis of silk protein.

Hendler (*4*, *5*) showed on hen oviduct tissue that amino acid uptake was almost abolished by the destruction of cell structures, whereas after the previous labelling of a minced tissue for a little while *in vitro*, the radioactivity was largely to be incorporated into the supernatant protein even in the homogenates in subsequent incubations.

On the other hand Butler *et al.* (*6*) and Gale (*7*) reported the facts that the incubation of labelled protoplast membrane of bacterium with unlabelled cytoplasm resulted in the labelling of cytoplasmic protein.

Though it remains obscure whether the cell debris fraction of silkgland corresponds functionally to the protoplast membrane of bacterium from a point of view already presented, an attempt was made to examine the possible involvement of cell debris fraction in the protein synthesis in cell free preparations of the posterior silkgland (*8*).

A fraction obtained from silkgland homogenates by low speed centrifugation at 700× *g* for 10 minutes was denoted as cell debri (CD)**. The prelabelled CD was highly radioactive and it was observed that under appropriate coditions, its radioactivity was transferred enzymatically to particulate protein. Furthermore, the radioactivity transferred into protein recognized above was of higher rate than that of the incorporation of C^{14}-glycine into protein.

In the present communication we report on the transfer of radioactivity from prelabelled CD to particulate fractions in the cell free preparations of posterior silkgrand, and its confirmation by dinitrophenylation technique.

Some factors influencing upon the transfer reaction *in vitro* have also been studied.

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* Some parts of this paper were reported at the 33rd annual meeting of the Japanese Biochemical Society (1960) and the 9th Symposium on the Nucleic Acids (1961), Tokyo.

** Abbreviations: ATP, adenosine triphosphate; GTP, guanosine triphosphate; tris, tris (hydroxymethyl) aminomethane; PCA, perchloric acid; TCA, trichloroacetic acid; CD, cell debris; RL, large particles, E_1, small particles; E_2, E_3, enzyme fractions; DNP-, dinitrophenyl-; FDNB-, 1-fluoro-2, 4-dinitrobenzene.

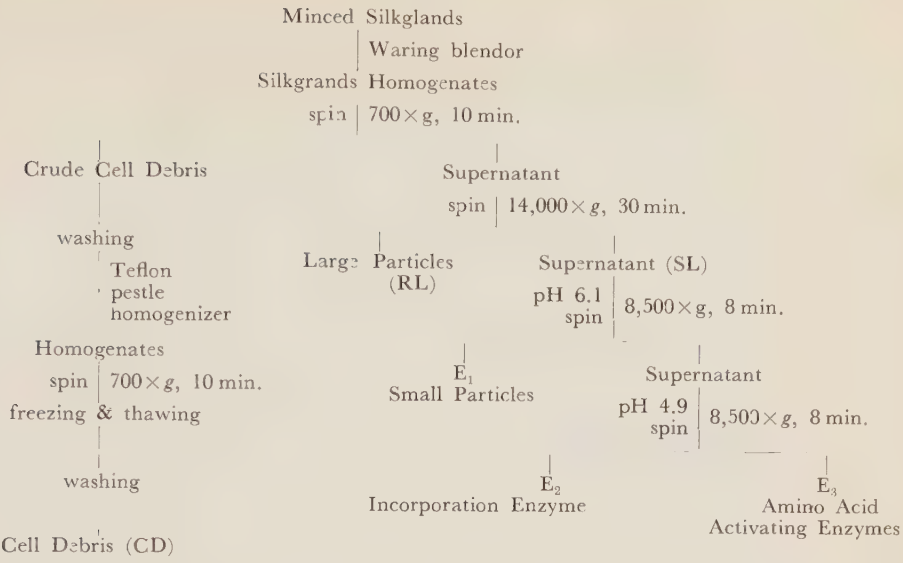

FIG. 1. Scheme for the subcellular fractionation of silkgland tissue.

1. Comparative studies on E_1 and so called microsomal (105,000×*g* precipitates) from the same tissue, performed by an electron microscopic examination and by an incorporation study of C^{14}-amino acids, identified E_1 and 105,000×*g* particles to be the same particulate fraction both morphologically and functionally*.

2. It is noted that E_2 contains the incorporation enzymes and E_3 includes amino acid activating enzymes (*10*, *11*).

MATERIALS AND METHODS

Animals—Domestic silkworms, *Bombix mori* L,, of both sexes, at the sixth to seventh day of fifth instar larvae were used.

Reagents and Chemicals—Adenosine triphosphate was obtained from Schwarz Bioreseach Inc., U. S. A. guanosine triphosphate from Sigma Chemical Co., U. S. A., and phosphocreatine from Calif. Biochemical Research, U. S. A. Crystalline pancreatic ribonuclease and deoxyribonuclease were purchased from Worthington Biochemical Corp., U. S. A. Crude creatine kinase was prepared by the method of Kuby *et al.* (*9*).

Radioactive amino acids, glycine-2-C^{14}, specific activity of 1.5 mc/mmole and glycine-1-C^{14}, 1.2mc/mmole were supplied from Daiichi Pure Chemicals Co., Tokyo. C^{14}-amino acids mixture (algal protein hydrolysate) was kindly issued by the Institute of Applied Microbiology, the University of Tokyo.

Fractionation of Silkgland Homogenates—Essentially Shimura's method (*10*) was applied (Fig. 1).

Preparation of Prelabelled CD (C^{14}-*CD*)—1) *Labelling of Silkglands—In vivo*: silkworms were injected 2 *μc per capita* of glycine-2-C^{14} and 15 minutes later the posterior silkglands were collected. *In vitro*: minced silkgrands were labelled with glycine-2-C^{14} for 15 minutes and collected. Incubation mixture consisted of: 4 g. of minced posterior silkgrands, 10 ml. of buffered 0.4 *M* sucrose at pH 7.4–7.6 (*10*), 10 *μc* of glycine-2-C^{14}. Incubations were run at 37°C for 15 minutes with constant shaking under oxygen as the gas phase.

2) *Fraction of CD*—Collected minces were washed thoroughly with cold buffer solution: twice with 10% glycine in buffered 0.4 *M* sucrose, three times with buffered 0.4 *M* sucrose. Washed minces then were homogenized in a Waring blendor for 60 seconds in four volums of buffered 0.4 *M* sucrose, filtered through double folded gauze. Filtrates were rehomogenized for 30 seconds more. Homogenates thus obtained were centrifuged at 700×*g* for 10 minutes, pellets were collected and rehomogenized with a teflon pestle. After the treatment of freezing, thawing and washing in buffered sucrose several times pellets were collected (Fig. 1). Microscopic observation of CD fraction revealed that the major components were nuclear debris and fragments of cell membranes without any intact cells (Fig. 2).

Incubation Procedures—A basal reaction mixture in the cell free preparations was shown in Table I.

* umpublished data

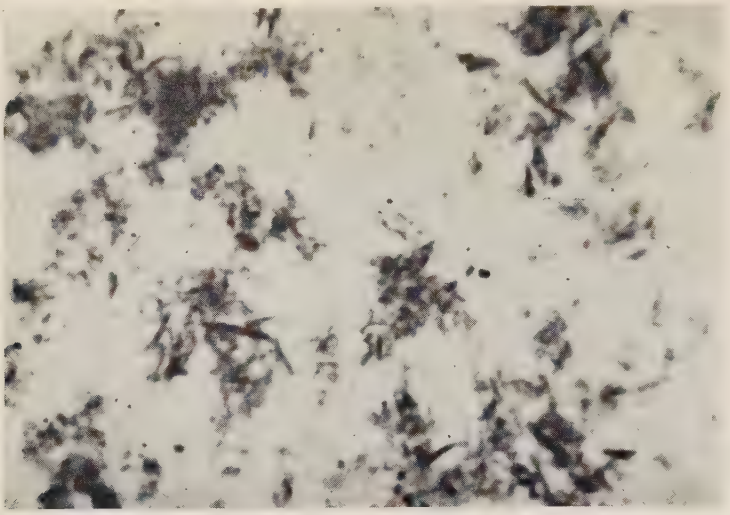

Fig. 2. Microscopic photography of CD (Giemsa staining, ×400)

At the end of the incubation period, the reaction mixture was chilled and an equal volume of cold 10% glycine solution was added, and shaken vigorously, CD as the C^{14}-source was removed by centrifugation at $1400 \times g$ for 10 minutes. All the fractionation procedures of particles from the supernates were performed in the cold, following the procedure shown in Fig. 1.

Table I

Constituents of the Reaction Mixture used in the Cell Free Systems

ATP	16 μmoles
GTP	0.5 μmoles
Phosphocreatine	20 μmoles
Creatine kinase	1.0 mg.
$MgCl_2$	8 μmoles
KCl	120 μmoles
Tris-HCl (pH 8.0)	100 μmoles
Sucrose	800 μmoles
19 amino acids mixture less glycine[1]	
CD	6–8 mg. of protein[2]
RL	4–8 〃
E_1	3–6 〃
E_2	3–6 〃
E_3	2–3 〃
C^{14}-CD total counts of	$4–6 \times 10^4$ c. p. m./vessel

final volume of 2.0 ml., pH 7.4–7.6.

1) Reaction mixture in 1 ml. contains 4 μmoles of DL-alanine and DL-serine; 0.75 μmole of DL-tyrosine; 0.5 μmole of L-valine, L-threonine, L-aspartic acid, L-asparagine, L-phenylalanine and L-glutamic acid; 0.25 μmole of L-isoleucine, L-histidine, L-leucine, L-proline and L-arginine; 0.125 μmole of L-glutamine, L-cystine, L-methionine, L-tryptophan and L-lysine (private communication of Dr. Shimura).

2) Protein content was determined by biuret reaction (*12*) using ovalbumin as a standard.

Extraction of Phospholipid, Phosphatidopeptide and Protein Fractions—The reaction was stopped by adding an equal volume of cold 8% PCA, subsequent extraction procedures were run according to the method of Huggins and Cohn (*13*), provided that PCA was used in place of TCA.

Preparation of Radioactive Samples—Lipid and phosphatidopeptide extracts were concentrated *in vacuo* while being slightly warmed and then plated on aluminium planchettes from micropipettes under the stream of warm air from a blower using a turn-table. Extracted protein was suspended in a small portion of water, plated and dried under an infra-red lamp.

Determination of Radioactivity—All the radioactive samples were counted in a windowless Q-gas flow counter, collected for self absorption in accordance with the study of Hendler (*14*). Results were expressed as specific activity (counts per minutes per mg.) with reference weight of 10 mg., unless otherwise stated. In all cases, sufficient counts were made to reduce the statistical error of counting to less than 5 per cent.

TABLE II

Transfer of Radioactivity from Prelabelled CD to Small Particles and Supernate Fractions

Incubation System	Incubation Time (min.)	Radioactivity transferred to			
		Small Particles			Supernate
		Lipid	Phosphatido-peptide	Protein	PCA-insoluble fraction
					c. p. m./mg.
Complete System ($E_1+E_2+E_3$)	0	4	1	23	9
〃	15	8	1	130	57
〃	30	10	0	114	62
〃	60	4	0	119	51
〃 $-E_3$	30	15	2	120	52
〃 —Amino acids mixture	30	0	3	53	47
〃 —GTP	30	6	1	96	55
〃 —ATP and ATP generating system	30	9	4	52	39

Complete systems consisted of: 16 μmoles ATP, 0.5 μmole GTP, 20 μmoles phosphocreatine, 1.0 mg. creatine kinase, 8 μmoles $MgCl_2$, 120 μmoles KCl, 100 μmoles tris-HCl (pH 8.0), 800 μmoles sucrose, 19 amino acids mixture less glycine, E_1 (5.8 mg. of protein), E_2 (7.8 mg. of protein), E_3 (3.2 mg. of protein), C_{14}-CD (547 c. p. m./mg.), total counts of 43,000 c. p. m./vessel, final volume of 2.0 ml. pH 7.4–7.6. Incubations were run at 37°C during the time indicated, with constant shaking.

RESULTS

1. *Transfer of Radioactivity from Prelabelled CD to Small Particles and Supernate Fractions*—Tabel II shows a time course of the transfer of radioactivity from prelabelled CD to particulate fraction, and conditions under which the transfer of radioactivity proceeded.

As can be seen from the time course, the transfer reaction was nearly completed in the first 15 minutes and showed no increment in further incubations.

Only a small incorporation was observed into lipid fractions.

On examining the effects of added cofactors, the omission of the amino acids mixture reduced the uptake by 44 per cent and the absense of ATP-energy generating systems caused the uptake to decrease by the same amount. GTP also seemed to be required in these transfer reactions. Omission of the E_3 fraction, amino acid activating enzymes, however, did not cause any inhibition upon the reaction. Therefore this appears to be unnecessary in the transfer systems.

The critical problem in such an experiment is whether the disintegration of CD occured during the reaction, or whether the labelled CD had contaminated the particulate and/or supernate fractions during the subsequent fractionation after the incubation.

In this point of view, the following experiment for confirmation was made.

2. *Incubation of Prelabelled CD Solely in the Buffered Medium*—As shown in Table III, so far as incubations were run in buffered sucrose, no radioactivity was detected in the cold PCA-soluble fraction of the supernate after the removal of C^{14}-CD from the reaction mixtures. If C^{14}-glycine was freed from C^{14}-CD it should appear in the cold PCA-soluble fraction.

The radioactivity recovered as the PCA-insoluble fraction was also negligible, *biz.*, at most 2 c. p. m. (Total counts), it took only about 0.03 per cent or less from the radioactivity which had been added at the beginning of the incubation.

Table III

Incubation of C^{14}-CD Solely in the Buffered Medium

Incubation Temperature	Incubation Time	Distribution of Radioactivity in the Supernate		
		Cold PCA-soluble	PCA-insoluble	%
	min.	total c.p.m.	total c.p.m.	
30°C	0	0	0	0
	15	0	0	0
	30	0	1.8	0.02
	60	0	0.8	0.01
37°C	15	0	0	0
	30	0	2.2	0.03
	30 (shaking)	0	0.6	0.01
	60	0	0	0

1. Reaction mixture consisted of : 800 μmoles sucrose, 120 μmoles KCl, 8 μmoles, $MgCl_2$, 100 μmoles tris-HCl (pH 8.0), 0.4 ml. C^{14}-CD suspension (total counts of 7,500 c. p. m./vessel), final volume of 2.0 ml., pH 7.4–7.6.

2. After the incubation C^{14}-CD was removed by centrifugation at 1,400×g for 10 minutes. An equal volume of cold 8% PCA was added to the supernatant. Cold PCA-soluble and PCA-insoluble fractions were separated, and total radioactivity counted.

It was consequently supposed that the actual contamination to lipid, phosphatidopeptide and protein fraction was almost negligible because of the complicated washing procedures through those extraction. Hence it would be reasonable to say that the results appearing in the preceding experiments are of no apparent incorporation caused by the contamination of C^{14}-CD to particulate proteins under such conditions as indicated elsewhere.

3. *Transfer of Radioactivity from Prelabelled CD to Particulate Fractions Consisted of RL*— Besides the E_1 particles which we used in the previous studies (*8*), the large particles were regarded as one of the main sites of protein synthesis in the posterior silkgland. That is, according to the experiments performed *in vivo* (*2*, *15*) or *in vitro* (*16*) using minced silkgland, RL showed a more marked protein synthesizing activity than the small particles (105,000×g particles or E_1), whereas it was suggested that both were microsomal in nature (*15*). And as will be shown later (*16*), the mitochondrial fraction which was naturally included in RL provided a minor influence on the transfer reaction through oxidative phosphorylation.

So we preferred to use the reaction systems consisting of RL in the following studies.

Table IV

Transfer of Radioactivity from C^{14}-CD to RL Systems

Incubation System	Distribution of Radioactivity	
	Phosphatidopeptide	Protein
		c.p.m./mg.
RL+E_1+E_2+E_3 (0-time)	0	57
RL+E_1+E_2+E_3	2	508
RL+E_1+E_2	2	515
RL	0	90

Reaction mixture consisted of the same components as described in Table I, except RL (4.7 mg. of protein), E_1 (4.4 mg. of protein), E_2 (8.6 mg. of protein), E_3 (2.5 mg. of protein), C^{14}-CD (1,058 c. p. m./mg.), total counts of 64,300c. p. m. /vessel; final volume of 2.0 ml., pH 7.4–7.6.

Incubations were run at 37°C for 30 minutes.

As shown in Table IV, the E_3 fraction was unnessary in these systems too.

TABLE V

Transfer of Radioactivity from C^{14}-CD to Particulate and Supernate Fractions

Incubation System	Incubation Time	Radioactivity transferred to			
		RL Fraction			Supernate (E_2+E_3)
		Lipid	Phosphatidopeptide	Protein	Protein
	min.				c. p. m./mg.
$RL+E_1+E_2+E_3$	0	0	0.6	56	—
//	5	1.6	2.3	222	8
//	10	1.1	1.2	168	8
//	15	1.3	0.4	187	11
//	30	—	0.7	169	11
//	60	0.6	0.2	302	12
				E_1-protein	
$E_1+E_2+E_3$	30	—	—	133	11

1. Reaction mixture consisted of the same components as shown in Table I, except RL (9.8 mg. of protein), E_1 (9.4 mg. of protein), E_2 (7.8 mg. of protein), E_3 (3.6 mg. of protein), C^{14}-CD (565 c. p. m. /mg.), total counts of 21,000 c. p. m./vessel; final volume of 2.0 ml., pH 7.4–7.6.

Incubations were run at 37°C for the times indicated.

2. As for the zero-time incubation, particles were not separated, but lipid, phosphatidopeptide and protein fractions were extracted from a whole supernate ($RL+E_1+E_2+E_3$).

Table V shows the time course of the transfer reaction, and a comparison between RL-system and E_1-system, reveals that the transfer reaction of radioactivity from C^{14}-CD to particulate protein was mostly completed within a short time.

Only a small incorporation of radioactivity into lipid fractions or supernatant protein was observed.

4. *Dinitrophenylation of RL after the Reaction*—To confim whether the transfer of radio activity from C^{14}-CD to particulate fractions is the net incorporation of radioactive substances into peptide chain or not, the RL separated after the reaction was dinitrophenylated and examined in the usual way.

At the end of the incubation period, RL was separated and suspended in distilled water and homogenized in a Potter-Elvehjem-type glass homogenizer. After treating it several times by freezing and thawing, it was dinitrophenylated (*17*), fractionated (*1*), and counted as shown in Fig. 3. Results were expressed as total c. p. m. in the fraction at infinite thinness.

It is generally said that fraction I contains DNP-derivatives of free amino acid and oligo-peptides; fraction II, DNP-derivatives of oligo- or poly-peptides; fraction III, DNP-derivatives of basic amino acids and peptides; fraction IV, DNP-derivatives of poly-peptides and mostly DNP-proteins (*1*).

As can be seen from Fig. 3, no radioactivity was detected in the other extracts (Fraction I), which indicate that the transfer of radioactivity observed above was not caused by a false reaction such as the adsorption of radioactive materials on the surface of particles.

DISCUSSION

It is generally believed in the mechanism of protein synthesis in the animal tissues that protein is synthesized through the steps proposed by Hoagland *et al.* (*18*):

1. Amino acid activation by its activating enzyme and amino acyl adenylate formation.

2. Transfer of activated amino acid to s-RNA.

3. Transfer of s-RNA-amino acid complex to ribosome.

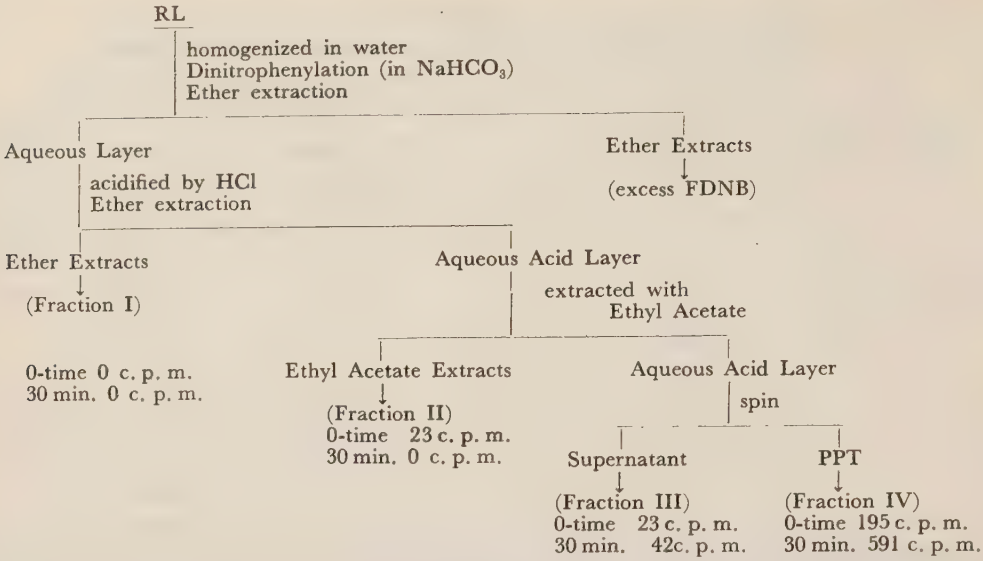

Fig. 3. Dinitrophenylation of labelled RL separated after the incubation. Constituents of incubation mixture was the same as described in Table I, provided that 0.4 ml. RL (8 mg. of protein), 0.4 ml. E_1+E_2 (5.2 mg. of protein), 0.4 ml. E_3 (0.6mg. of protein), 0.3 ml. C^{14}-CD (375 c.p.m./mg., total counts of 22,700 c. p. m./vessel); final volume of 2.0 ml., pH 7.4–7.6.

Incubations were run at 37°C, for 30 minutes, with constant shaking.

4. In turn becomes a part of, or passes its amino acid to the template for incorporation into the protein.

Although relatively little is known about the similar mechanism in insects, there has been considerable doubt on the problem that the silk protein is synthesized through the process of "conventional" protein synthesis as referred to above.

While Ochoa (*19*) reported the existence of amino acid "incorporation" enzymes in *Alcaligenes faecalis*, Shimura *et al.* (*10*, *11*) also reported that at least two kinds of enzymes were involved in the incorporation of C^{14}-glycine into the particulate proteins. One is the enzyme which participates in the amino acid activation, and the other contributes to the incorporation of C^{14}-glycine into the particulate fractions.

The amino acid depending PP-exchange reaction was not correlated with the incorporation of C^{14}-amino acid into particulate protein. Accordingly they assumed that silk protein might be synthesized not through a pathway which regards the amino acyl adenylate as the starting point proposed by Hoagland *et al.*, but through another way in the silkworm's posterior silkgland (*20*).

Hendler (*4*, *5*) showed on hen oviducts that it lost virtually all its ability to incorporate free amino acid into protein upon homogenation, as silkworm's posterior silkgland. Homogenation of a prelabelled mince, however, followed by subsequent incubation, has led to the labelling of a considerable portion of supernatant protein. From this observation he suggested the existence of an intermediate stage, between free amino acid and protein, which scarcely is formed in the homogenate, but once formed in the intact cell serves as a source of radioactivity for the protein.

In bacteria, it is said that the protoplast membrane is labelled most quickly and by subsequent incubation with nonlabelled cytoplasm, the cytoplasmic protein as a result becomes radioactive (*6*, *7*). Gale (*7*) has confirmed this problem on *Staphylococcus aureus*: that there exists a precursor of protein in the protoplast membrane, and by successive incubation it can be incorporated into the cell wall peptide or the cytoplasmic protein. He

TABLE VI

In vivo Incorporation of Glycine-1-C^{14} into Subcellular Fractions of Posterior Silkglands during 15 Minutes

Cellular Fraction	Distribution of Radioactivity		
	Lipid	Phosphatidopeptide	Protein
			c. p. m./mg.
CD	16	54	9800
RL	7	25	7670
E_1	4	5	2350
E_2	4	7	653
E_3	3	17	88

Silkworms were injected 2 μc *per capita* of glycine-1-C^{14} and 15 minutes later the posterior silkglands were collected. The technique of preparing the cellular fractions from the posterior silkgrands was described in the previous section.

assumed from these results that the initial stage of protein synthesis might occur in the protoplast membrane.

As a matter of fact, also in the posterior silkgland, CD fraction was labelled so rapidly (Table VI) that it suggested the same situation.

Although Hendler (*5*) has indicated lipids as the intermediate carrier in protein synthesis, and Huggins & Cohn (*13*) regarded phosphatidopeptide as a precursor of protein, with above considerations in mind, our previous studies (*8*) on the protein synthesis of the posterior silkgland have been proceeded. And a marked protein synthesis was recognized in the cell free-systems applying the prelabelled CD as the C^{14}-source. Namely, the incorporation rate* of C^{14}-glycine into protein fraction of reconstructed homogenate (CD+RL+E_1+E_2+E_3) was about 0.2 per cent; C^{14}-glycine into particulate protein in a reaction system of RL+E_1+E_2+E_3 was around 0.05 per cent; whereas transfer of radioactivity from C^{14}-CD to RL-protein (an incubation system of RL+E_1+E_2+E_3) was around 5.2 per cent; and C^{14}-CD to E_1-protein (an incubation system of E_1+E_2+E_3), 1.5 per cent.

In this paper, the transfer of radioactivity from prelabelled CD to particulate proteins were shown and confirmed by the dinitrophenylation study of RL isolated after the incubation.

Thus it might be postulated that radioactive substances existing in cell debris can be regarded as one of the precursors of protein in the posterior silkgland.

* Incorporation rate = total c. p. m. incorporated/total c. p. m. of C^{14}-source.

SUMMARY

The investigation of the transfer of radioactivity from prelabelled cell debris to particulate fractions in the cell-free preparations of the posterior silkgland was developped.

1. After labelling of silkglands with C^{14}-glycine for 15 minutes *in vivo* or *in vitro*, posterior silkglands were collected and homogenized. The cell debris fraction was obtained by centrifugation at 700×g for 10 minutes. Microscopic observation of cell debris revealed that major components were nuclear debris and fragments of cell membranes without any intact cells.

2. Prelabelled cell debris thus prepared was highly radioactive and it was shown that under appropriate conditions, the radioactivity was transferred enzymatically to particulate protein. This was confirmed by dinitrophenylation study of isolated particles after the reaction.

3. Radioactivity was principally incorporated into protein of particulate fractions, but not very much into lipid and supernatant fractions.

4. As regards the transfer reaction, amino

acid activating enzyme (E_3) seemed to be unnecessary, whereas amino acids mixture, GTP, ATP and ATP-generating systems were required.

The work has been supported by grants partly from the Ministry of Education to the research group for Fibroin Biosynthesis and partly from the Rockefeller Foundation to one of the authors. We wish to thank Dr. K. Shimura and his staff for their valuable discussion and Dr. B. Maruo for his kind supply of C^{14}-all-labelled amino acids.

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Ribonuclease T_1 Digestion of Yeast Soluble RNA*

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(Received for publication, July 28, 1961)

The active role of tansfer or soluble RNA (s-RNA) in protein synthesis is generally accepted. The attitude of s-RNA towards various enzymes attacking RNA such as polynucleotide phosphorylase (*1*, *2*) and pancreatic RNase (*3*) has been studied. The latter liberates aminoacyl adenosine from amino acid-charged s-RNA. This report deals with the digestion of yeast s-RNA by RNase T_1 with special references to the liberation of amino acid-charged oligonucleotides from C^{14}-labeled amino acid-charged s-RNA.

MATERIALS AND METHODS

s-RNA—s-RNA was prepared from fresh pressed baker's yeast purchased from a local store essentially according to the Monie's modification (*4*) of Kirby's method.

RNase T_1—Prepared from Takadiastase Sankyo by the modified method of Takahashi (*5*) of the original method by Sato and Egami (*6*). Following are all commercial products.

ECTEOLA-cellulose	(SERVA Entwicklungslabor, Heidelberg)
DEAE-cellulose	(SERVA Entwicklungslabor, Heidelberg)
Sephadex G-50	(PHARMACIA Uppsala, Sweden)
Yeast RNA	(C. F. Boehringer and Soehne GmbH, Mannheim)

C^{14}-Labeled Amino Acid-charged s-RNA—It was prepared essentially according to Osawa (*7*). 234 mg. purified s-RNA, 25μC of Chlorella protei n hydrolysate (4.9 mC/m mole C), 50 mg. of non-labeled 18 amino acids mixture (glycine, alanine, valine, leucine, tyrosine, phenylalanine, proline, hydroxyproline, serine, threonine, methionine, cystine, glutamic acid, aspartic acid, histidine, arginine, lysine and tryptophan, *ca* 30 μmoles respectively), 40 ml. of dialyzed 105,000$\times g$ supernatant fraction of yeast cell-free extract, 500 μmoles of ATP, 10 ml. of 0.2 *M* Tris buffer (pH 7.6) containing 10^{-3} *M* Mg^{++}, adjusted to the final volume of 100 ml. was incubated for 15 minutes at 37°C. Then an equal volume of 90% phenol was added to the reaction mixture. It was stirred for one hour and centrifuged. Water layer was collected, and 2 volumes of alcohol was added. Then it was kept in a deep-freezer for 2 hours. The precipitate was collected, dissolved in 0.03 *M* Tris buffer (pH 6.8) containing 0.1 *M* NaCl, dialyzed against the same saline followed by distilled water and lyophilyzed.

C^{14}-labeled amino acid-charged s-RNA (190 mg.) was obtained, and 1 mg. of the product has a radioactivity 608 c. p. m. (*ca* 2.2 μg. amino acids mixture).

EXPERIMENTAL

1) *Course of Digestion of s-RNA by RNase T_1*—As shown in Table I, the digestion rate of s-RNA by RNase T_1 is far slower than that of commercial yeast RNA, although finally the former was as well digested as the latter. The result is consistent with that of G. L. Cantoni, who showed that s-RNA of rabbit liver was digested quite well by RNase T_1 as expected from the specificity (Personal communication).

The initial relative resistance of s-RNA towards RNase T_1 might be related to the special configuration of s-RNA. It should be pointed out that s-RNA is known to be highly resistant to polynucleotide phosphorylase (*1*, *2*).

* Abbreviations: RNA, ribonucleic acid; RNase, ribonuclease; s-RNA, soluble RNA; Tris, tris (hydroxymethyl) aminomethane; DEAE-cellulose, diethylaminoethyl-cellulose; G, guanosine; A, adenosine; C, cytidine; U, uridine; p, phosphoryl group bound to the 3′-hydroxyl group of the left nucleoside and to the 5′-hydroxyl group of the right nucleoside.

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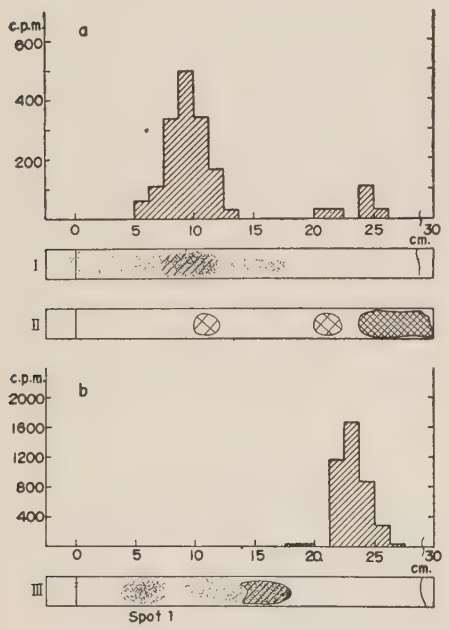
Table I

RNase T_1 Digestion of Yeast s-RNA

Substrate	RNase T_1 units	2.93	293
	Reaction time	15 min.	6 hours
	ΔOD_{260}		soluble P
			total P
RNA		0.293	48%
s-RNA		0.072	52
C^{14}-amino acid-s-RNA		0.098	50

Reaction mixture (=1 ml.) which contains 3 mg. of substrate, RNase T_1, final 0,002 *M* EDTA and final concentration of 0.04 *M* Tris buffer (pH 7.5) was incubated at 37°C. Activity was measured by the method described in previous reports (*6*). One unit of RNase T_1 is an amount of the enzyme which produces $\Delta OD_{260}=0.1$ in 15 minutes.



: UV absorption,
: fluorescence in acid vapour*
: ninhydrin positive

Fig. 1. Paper chromatograms of the digestion products of C^{14}-amino acid-charged s-RNA by RNase T_1.

I. Aliquot of the reaction mixture (=20 ml.) which contains 150 mg. of C^{14}-amino acid-charged s-RNA, 200 μg. of RNase T_1, 2 ml. of 0.1 *M* Tris buffer (pH 7.5) and incubated for 6 hours at 37°C was subjected to an ECTEOLA-cellulose column (bufferized with 0.01 *M* ammonium bicarbonate, pH 7.0) and the fraction which passed through the column without adsorption was chromatographed after lyophilization.

a) The radioactivity measured after cutting off the paper shown in I.

II. Paper chromatogram of free amino acids mixture.

III. Paper chromatogram of the digestion mixture (same as in I) after treatment with alkali (pH 10, 37°C, 15 min.)

b) Radioactivity of the paper (shown in III). The spot 1 was shown to contain adenosine and cytidine as the nucleoside by the absorption spectra.

Solvent: saturated ammonium sulfate: water: isopropanol (79 : 19 : 2) (*8*)

* It shows the position of guanine derivatives; on this paper they are 2′, 3′-cyclic guanylic acid and 3′-guanylic acid.

2) Separation and Identification of C^{14}-labeled Amino Acid-charged Oligonucleotide Liberated from Amino Acid-charged s-RNA by RNase T_1.

Amino acid-charged s-RNA was digested by RNase T_1 in the conditions described under the Fig. 1. The reaction mixture was subjected to an ECTEOLA-cellulose column (0.01 *M* ammonium bicarbonate, pH 7.0) to separate from a bulky amount of oligonucleotides produced by the RNase T_1 digestion, and the fraction which passed through the column without adsorption was chromatographed on paper after lyophilization (shown

in Fig. 1). The paper cut off and its radioactivity was measured. Then the same fraction (shown in I and a) was chromatographed after alkaline treatment (III and b). The UV-absorbing region on the paper chromatogram (I) had a high radioactivity (a). After alkaline hydrolysis, it is shown that amino acids were liberated from amino acid-charged oligonucleotides (III, b). The oligonucleotide produced by alkaline treatment (Spot 1 in III) was shown to contain adenosine and cytidine by measuring ultraviolet absorption spectra.

In order to identify the amino acid-charged oligonucleotide the reaction mixture (same as in the case of Fig. 1) lyophilyzed was subjected to gel filtration with Sephadex G-50 column. Elution courves (Fig. 2) with distilled water pursued by measuring radioactivity and optical density at 260 mμ showed the existence of C^{14}-amino acid-charged oligonucleotides (peaks 1, 2, 3, 4) besides C^{14}-amino acids (peaks 5, 6).

Shadowed region (peak 4) in Fig. 2, which seems to consist mainly of amino acid-charged oligonucleotides. was subjected to DEAE-cellulose column chromatography (Fig. 3). Shadowed fraction was collected and analyzed. The shadowed fraction (Fig. 3) seemed to be the main fraction of amino acid-charged oligonucleotides. Finally the nucleotide composition of the fraction was analyzed by usual acid hydrolysis and the amino acids content was calculated from radioactivity. It was found that the fraction contained adenine and cytosine with the molar ratio of 8 : 7 and a trace of uracil. And the weight ratio of nucleotides to amino acids was calculated to be 12.3. Because the nature of amino acids charged to the oligonucleotide had not been identified, only an approximate calculation for the molar ratio could be achieved. Assuming the molecular weight of the amino acids to be 100 or a little more, the ratio may be calculated as 4–5. So taking the results and the specificity of RNase T_1 (*9*)

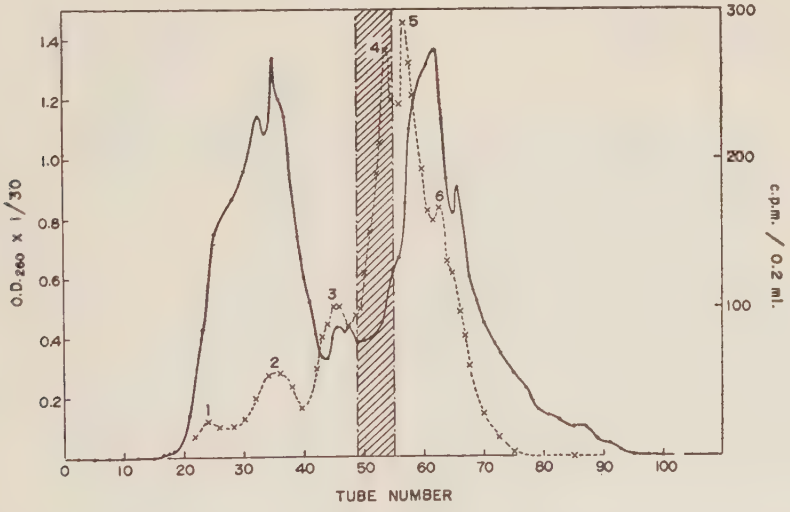


Fig. 2. Sephadex G-50 column chromatography of RNase T_1 digestion mixture of C^{14}-labeled amino acid-charged s-RNA from yeast. Sephadex G-50, 1.8×40 cm. Reaction mixture was the same as in the Fig. 1. Incubation: 6 hours at 37°C. Elution with distilled water, one tube contains 1.9 ml.

——: optical density at 260 mμ
- - - : radioactivity

Shadowed region is collected and subjected to the DEAE-cellulose column (shown in Fig. 3).

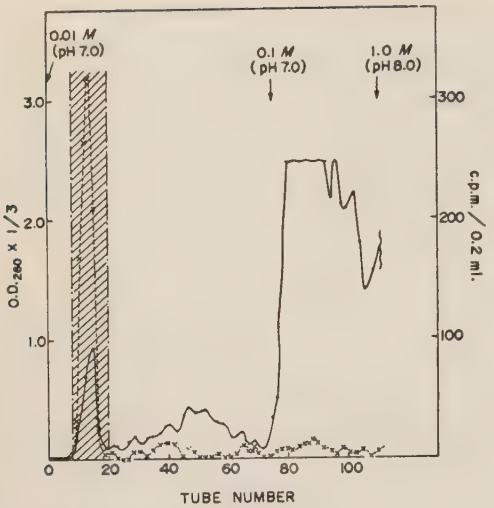


Fig. 3. Separation of C^{14}-amino acids-charged oligonucleotide from oligonucleotides mixture produced by RNase T_1 digestion of C^{14}-amino acid-charged s-RNA.

DEAE-cellulose column, 0.8×15 cm., was bufferized with 0.01 *M* ammonium bicarbonate (pH 7.0). Shadowed region (in the Fig. 2) was subjected to the column after adding ammonium bicarbonate buffer to final 0.01 *M* (pH 7.0).

——: optical density at 260 mμ

- - - : radioactivity

into consideration, it is suggested in accordance with the well-established fact (*10*) that the amino acid terminal nucleotide sequence in the fraction may be—GpApCpCpA-amino acid.

It is generally considered that s-RNAs are amino acid specific and each amino acid specific s-RNA has a specific nucleotide sequence. The experiments described here, although preliminary, suggest that the amino acid terminal nucleotide sequence or at least that of one of the main components of yeast s-RNAs may be—GpApCpCpA. The result seems to be consistent with that of Canellakis (*11*).

SUMMARY

1. Yeast s-RNA was slowly, but finally as well digested as commercial yeast RNA by RNase T_1

2. Amino acid-charged oligonucleotide was obtained by the digestion of C^{14}-labeled amino acid-charged s-RNA by RNase T_1.

3. The existence of an amino acid terminal nucleotide sequence (—GpApCpCpA-amino acid) was suggested.

The authors are indebted to Dr. S. Osawa of Faculty of Science of Nagoya University for his helpful advice and to Sankyo Pharmaceutical Co. Ltd. for the gift of Takadiastase Sankyo.

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The Journal of Biochemistry, Vol. 50, No. 5, 1961

The Presence of Adenylyl 2,3-Diphosphoglyceric Acid in Human and Rabbit Blood

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(Received for publication, June 16, 1961)

The isolation and identification of adenylyl 2,3-diphosphoglyceric acid in pig blood has been reported in previous paper (*1*, *2*). The presence of this nucleotide in human blood has been discussed (*2*), because other workers (*3*) have found a minor unknown adenine nucleotide in human blood which behaved very similarly to adenylyl 2,3-diphosphoglyceric acid on Dowex 1-formate column chromatography. This communication deals with the isolation of adenylyl 2,3-diphosphoglyceric acid from human and rabbit blood.

Human and rabbit blood were extracted with ice-cold perchloric acid and the phosphorus compounds were precipitated from the extracts by the addition of barium acetate. After removal of barium with Amberlite IR 120-H^+, the phosphate compounds were chromatographed on Dowex 1-formate (X10) column using the modified formic acid system (*2*). The chromatograms, given in Fig. 1, suggest that the new nucleotide (X) is present in these bloods. The nucleotide fractions were collected and treated with charcoal (Shirasagi A, previously washed with ethanolic octyl alcohol) to separate the nucleotide completely from any contaminating 2,3-diphosphoglyceric acid. The charcoal eluates were run through a column of Dowex 1-Cl and, after the column was throughly washed with 0.1 *M* NH_4Cl, the nucleotide was eluted by 0.1 *N* HCl as described in the previous paper (*2*).

The ultraviolet absorption spectra at different pHs of the nucleotide obtained from these blood were those characteristic of an adenine nucleotide and coincided with that of pig adenylyl 2,3-diphosphoglyceric acid.

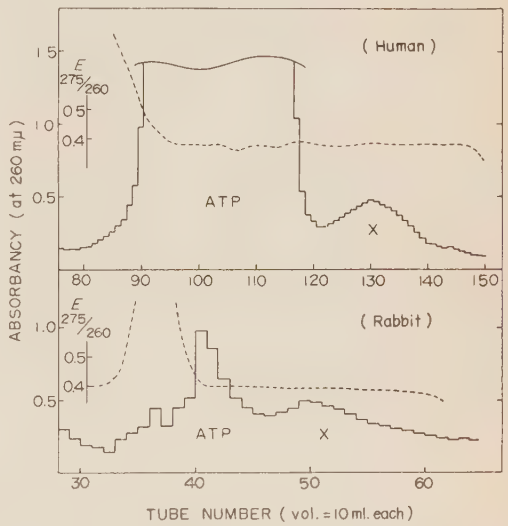

Fig. 1. Separation of adenylyl 2,3-diphosphoglyceric acid by Dowex 1-formate column chromatography.

Nucleotides from 3 liters of human blood[1], after purification by absorption to and elution from charcoal, was applied on a 1 × 20 cm. column. Rabbit blood used was 500 ml. In this case the charcoal treatment was omitted because of its smaller content of total phosphates. Elution was started with water (300 ml. for the rabbit sample or 500 ml. for the human sample) in mixing flask and 4 *N* formic acid-0.4 *M* ammonium formate in the reservoir. The new nucleotide (X) was eluted just after ATP.

1) Human blood was furnished from the Blood Transfusion Service, Tokyo University Hospital.

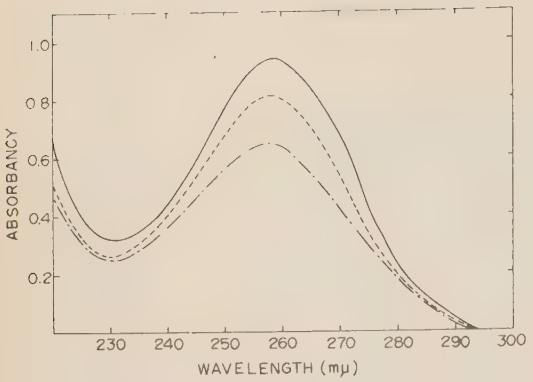

Fig. 2. Ultraviolet spectra of the nucleotide in acid obtained from blood of various animals.

Human nucleotide (----), rabbit nucleotide (—·—) and pig blood adenylyl 2,3-diphosphoglyceric acid (——).

The spectra of the nucleotide obtained from different sources in acid solution, are shown in Fig. 2. When chromatographed on paper with four different developing solvents (*4*)– *n*-butyric acid-NaOH, *n*-butyric acid-NH_4OH, *n*-propanol-ammonia-water, and *n*-propanol-phosphate buffer-ammonium sulfate—, the nucleotide behaved differently from ATP and was located at the same R_f's as that of the pig one. Analytical data of the composition of the nucleotide from both human and rabbit blood, were also the same as that of the pig adenylyl 2,3-diphosphoglyceric acid. Table I shows that one mole of the nucleotide had one mole ribose, one mole glyceric acid and three moles phosphoric acid but no other labile phosphoric acid residues. AMP and 2,3-diphosphoglyceric acid derived from the nucleotides by the action of snake venom nucleotide pyrophosphatase (*5*), were identified enzymatically with the use of adenylate deaminase and phosphoglyceric acid mutase-enolase system respectively (*2*).

The content of the nucleotide was about one per cent of the total nucleotides in human blood, and about three per cent in rabbit blood. But we failed to find it in cow blood which have been shown to be markedly different from human, rabbit and pig blood in its very small content of 2,3-diphosphoglyceric acid.

It is interesting that the nucleotide was found only in bloods which have been known to contain a large amount of 2,3-diphosphoglyceric acid.

Table I

Analyses of Nucleotides Obtained

Analytical values are expressed as moles per mole of adenine. The molar extinction coefficient of the nucleotide at 260 mμ in acid was taken as 14,300. Ribose was determined by the orcinol reaction (*6*), phosphorus by the method of Berenblum and Chain (*7*), and glyceric acid by the method of Bartlett (*8*) using 2,3-diphosphoglyceric acid as the standard.

Nucleotide obtained from	Adenine	Ribose	Labile P	Total P	Glyceric acid
Pig[1)]	1.00	0.98	0.15	3.04	0.99
Human	1.00	1.02	0.23	2.77	0.97
Rabbit	1.00	0.91	0.24	3.12	0.95

1) The data has been reported in the previous papers (*1*, *2*).

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PROGRAM

for

The 34th Annual Meeting of the Japanese Biochemical Society

November 4～6, 1961

Held at Faculty of Medicine,
Osaka University, Osaka

The Japanese Biochemical Society

November 4th (Saturday)

Room A

Morning Session

General Topics

9 : 00 (A- 1) Substrate specificity of phosphomonoesterases from several plant sources—Tomoyoshi IKAWA,* Kazutosi NISIZAWA & Tomo-o MIWA (Bota. Inst., Fac. of Sci., Tokyo Kyoiku Univ.)

9 : 15 (A- 2) Biochemical studies on nivalin and galanthamin, with special references to their effects on cholinesterase—Junji KIKUCHI,* Hiroyoshi MURATA, Reiji HUJITA & Yozo MATSUBARA (Dept. of Biochem., Tokyo Med. Coll.)

9 : 30 (A- 3) Enzymatic hydrolysis of nitrophenol-fatty acid esters—Kei FURIYA & Shin FURIYA (Dept. of Biochem., Tokyo Women's Med. Coll. & Dept. of Biochem., Med. Sch., Chiba Univ.)

9 : 45 (A- 4) Purification of phosphomonoesterase in dental pulp—Tadao KIMIZUKA* & Atushi OSHIKANE (Dept. of Biochem., Sch. of Dent., Nihon Univ.)

10 : 00 (A- 5) Low-molecular-weight substrates for lysozyme—Tomojiro SEKI,* Koichi IWAI & Toshio ANDO (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

10 : 15 (A- 6) Purification and some properties of phytase from wheat bran (III)—Yasutoyo NAGAI* & Saburo FUNAHASHI (Dept. of Agric. Chem., Univ. of Tokyo)

10 : 30 (A- 7) Effect of substrate analogues on RNase-IA in γ-irradiation—Keizo WAKU* & Tyunosin UKITA (Fac. of Pharm. Sci., Univ. of Tokyo)

10 : 45 (A- 8) Enzymic delactonization of dehydroascorbic acid—Hisashi TAKIGUCHI* & Yasuo KAGAWA (Dept. of Biochem., Fac. of Med., Univ. of Tokyo)

11 : 00 (A- 9) Studies on soluble lactonases—Minoru KAWADA, Kantaro SUZUKI, Yasuo KAGAWA & Hisashi TAKIGUCHI (Dept. of Biochem.. Fac. of Med. Univ. of Tokyo)

11 : 15 (A-10) Effect of monovalent cations on the enzymatic activity of the B component of *E. coli* tryptophan synthetase—Masakazu HATANAKA,* Kengo HORIBATA & I.P. Crawford (Dept. of Med. Chem., Sch. of Med., Kyoto Univ.)

11 : 30 (A-11) Inactivation of enzyme under high pressure : Trypsin—Keizo SUZUKI, Kinjiro MIYAGAWA* & Kazuhiro HIROYAMA (Fac. of Sci. Tech., Ritsumei Univ. & Osaka Jyoshigakuen Junior Univ.)

11 : 45 (A-12) Hydrolysis of B-chain of insulin and glucagon by *Mamushi* venom proteinase—Yomoji SUZUKI, Sadaaki IWANAGA, Masatoshi SATAKE* & Tamotsu ŌMORI (Fac. of Pharm. Sci., Kyoto Univ.)

12 : 00 (A-13) On *Streptomyces* leucine aminopeptidase—Takeshi ŌUCHI* & Akira HIRAMATSU (Biochem. Lab., Fac. of Agric., Ibaraki Univ.)

12 : 15 (A-14) The active center of carboxypeptidase-A—Hideko FUJIOKA,* & Kazutomo IMAHORI (Dept. of Chem., Coll. of General Educ., Univ. of Tokyo)

Afternoon Session

General Topics

1 : 30 (A-15) A new colorimetric determination method for iminopeptidase and changes of the enzyme activity in some organs of rat and chick embryo—Yosoji ITO, Katsumi WAKABAYASHI,* Hiroyoshi ENDO & Hiroshi ENOMOTO (Dept. of Physiol. Chem., Fac. of Pharm. Sci., Univ. of Tokyo)

1 : 45 (A-16) Enzymatic hydrolysis of antibiotic cyclic peptide. (II) Hydrolysis of colistin—Gen Isshiki*, Yoshitake Saito & Shohei Otani (Dept. of Biochem., Med. Sch., Osaka City Univ.)

2 : 00 (A-17) Enzymatic hydrolysis of antibiotic cyclic peptide. (I) Hydrolysis of gramicidin S—Munehiko Yukioka,* Yositaka Saito & Shohei, Otani (Dept. of Biochem., Med. Sch., Osaka City Univ.)

2 : 15 (A-18) Partially purified hydazidase (isoniazid-decomposing enzyme)—Ichiro Toida (Res. Inst. of Tuberc., Japan Anti-Tuberc. Associ.)

2 : 30 (A-19) Studies on nicotinamidase from *Mycobacteriaceae*—Koji Suzuki & Tokuji Kimura (Lab. of Biochem., St. Paul's Univ.)

2 : 45 (A-20) Formamidase in *Mycobacteria*: its enzymatic properties—Hideo Nagayama, Kiyoshi Konno & Sutemi Oka (Res. Inst. for Tuberc. & Leprosy, Tohoku Univ.)

3 : 00 (A-21) Conversion of prothrombin into thrombin. (III) Purification of prothrombin and thrombin—Yuzaburo Masaki*, Hiroaki Kinjo & Toshio Asada (Dept. of Biochem., Sch. of Med., Toho Univ.)

3 : 15 (A-22) On the substrate specificity of bacterial ε-lysine acylase—Ichiro Chibata, Tsutomu Ishikawa & Tetsuya Tosa* (Osaka Res. Lab., Tanabe Seiyaku Co.)

3 : 30 (A-23) Kynurenine transaminase from horse kidney—Yumiko Ueno*, Sachiko Iimura & Ryoichi Shukuya (Dept. of Biochem., Nippon Med. Sch.)

3 : 45 (A-24) Further purification of kynurenin transaminase and its properties—Nobuaki Ogasawara*, Yasumichi Hagino & Yahito Kotake (Dept. of Public Health, Sch. of Med., Nagoya Univ.)

4 : 00 (A-25) Study on nature and biochemical role of mitochondria bound transaminase—Nobuhiko Katunuma* & Makoto Matuda (Enz. Chem., Sch. of Med., Tokushima Univ. & Inst. for Protein Res., Osaka Univ.)

4 : 15 (A-26) Some properties of the reductoisomerase formed by mutants of *Neurospora crassa*—Kazuyoshi Kiritani (Inst. of Applied Microbiol., Univ. of Tokyo)

4 : 30 (A-27) Enzymatic transglucosylation with isomaltose as substrate—Keiko Ishizawa* & Tomo-o Miwa (Dept. of Agric. & Dept. of Bota., Fac. of Sci., Tokyo Kyoiku Univ.)

4 : 45 (A-28) The enzymic formation of glyoxylic acid from malic acid by extracts from *Rhodopseudomonas spheroides*—Syōzō Tuboi* & Goro Kikuchi (Dept. of Biochem., Sch. of Med., Tohoku Univ.)

5 : 00 (A-29) On the decarboxylase found in the degradative pathway of L-leucine—Shoji Sasaki (Dept. of Bota., Fac. of Sci., Hokkaido Univ.)

November 4th (Saturday)

Room B

Morning Session

General Topics

9 : 00 (B- 1) Chemical analysis of arterial wall. (I) Mucopolysaccharides of human arterial tissue—Noriko Yabuta, Kazuhiro Tamari, Motoomi Nakamura & Tamaki Imai (Dept. of Patho., Res. Inst. of Angiocardiol., Med. Sch., Kyushu Univ.)

9 : 15 (B- 2) An acid mucopolysaccharide from amyloid-laden spleen—Takashi Okuyama,* Koh-iti Turumi & Kazuko Kikuta (Depts. of Biochem. & Pathol., Fukushima Med. Coll.)

9 : 30 (B- 3) Neutral glycans in connective tissues and their changes with ageing—Senitiroh Hakomori,* Kimikiyo Kato & Hiroaki Kawauti (Cancer Inst., Tohoku Pharm. Univ.)

9 : 45 (B- 4) The actions of the proteolytic enzyme "Pronase" on blood group-specific mucopolysaccharides—Kazu Hashimoto* & Noboru Hiyama (Dept. of Biochem., Fac. of Med., Hirosaki Univ.)

10 : 00 (B- 5) Some oligosaccharides isolated after acetolysis of the blood group B mucopolysaccharide from human ovarian cyst fluid—Teruji Sasaki* & Noboru Hiyama (Dept. of Biochem., Fac. of Med., Hirosaki Univ.)

10 : 15 (B- 6) On sialic acid of *Escherichia coli*. (I)—Atsushi Kimura* Takashi Okuyama (Dept. of Biochem., Fukushima Med. Coll.)

10 : 30 (B- 7) Studies on the type-specific polysaccharides from certain Mucoid strains of *E. coli*. (V) Preparation of M–I and M–II polisaccharides and their immunochemical properties—Shuzo Akashi, Hideo Goto, Shigeko Inoue, Akinobu Tsuyama & Minoru Sasaki* (Dept. of Biochem., Med. Sch., Nagoya City Univ.)

10 : 45 (B- 8) Studies on the type-specific polysaccharides from certain mucoid strains of *E. coli*. (IV) On the determinant group of the polysaccharide (Ps-I) participating in the specific precipitin reaction—Shuzo Akashi & Hideo Goto* (Dept. of Biochem., Med. Sch., Nagoya City Univ.)

11 : 00 (B- 9) Accumulation of UDP-aminosugar in *Staphylococcus aureus*—Eiji Ito* & Masahiro Saito (Dept. of Chem., Fac. of Sci., Hokkaido Univ.)

11 : 15 (B-10) Accumulation of CDP-polyol in *Staphylococcus aureus*—Masahiro Saito* & Eiji Ito (Dept. of Chem., Fac. of Sci., Hokkaido Univ.)

11 : 30 (B-11) Amino sugars in the defatted cells of *Bacillus tuberculosis*—Kimiko Anno & Noriko Yoshimoto* (Dept. of Chem., Fac. of Sci., Ochanomizu Univ.)

11 : 45 (B-12) Biochemical studies on hexosamines. (XI) Talosamine—Shoji Fujii* & Hideo Kushida (Res. Lab., Kaken-Yaku-Kako Co.)

12 : 00 (B-13) Studies on mucopolysaccharides. (III) The hexosaminide linkage in shark chondroitin sulfate—Konoshin Onodera & Tohru Komano* (Biochem. Lab., Coll. of Agric., Kyoto Univ.)

12 : 15 (B-14) Enzymatic digestion of chondroitin sulfate and chromatographic separation of the digestion products—Yukio Miyakoshi, Kazuaki Sakai & Ikuo Yamashina (Chem. Dept., Fac. of Sci., Kanazawa Univ.)

Afternoon Session

General Topics

1 : 30 (B-15) The nature of linkage between placetin and galactose—Edahiko Murakami & Ayako Hisatake (Dept. of Chem. & Domes. Sci., Nagoya Branch Sch., Aichi Gakugei Univ.)

1 : 45 (B-16) Studies on metabolism of L-rhamnose by *Escherichia coli*—Hideo Sawada, Masako Kadoya & Yasuyuki Takagi (Dept. of Biochem., Med. Sch., Kanazawa Univ.)

2 : 00 (B-17) Inosine metabolism in human erythrocytes. (II) Identification of the intermediates—Yoshinari Ishii, Takashi Hashimoto, Masamiti Tatibana &

Haruhisa Yoshikawa (Dept. of Physiol. Chem. & Nutr., Fac. of Med., Univ. of Tokyo)

2: 15 (B-18) Incorporation of glucose into starch by soybean seedling—Toshio Fukui & Ziro Nikuni (Inst. Sci. & Ind. Res., Osaka Univ.)

2: 30 (B-19) On the lactosin of *Dianthus superbus*—Susumu Murakami (Dept. of Biol., Saitama Univ.)

2: 45 (B-20) Determination of vitamin B_6 derivatives—Isao Nakahara,* Yoshimasa Morino & Hiroshi Wada (Dept. of Biochem., Med. Sch., Osaka Univ.)

3: 00 (B-21) Relationship between the toxicity of L-cysteine and its inhibitory action on B_6-dependent enzyme—Masami Suda, Akira Kato,* Nobuhiko Katsunuma & Makoto Matsuda (Inst. for Protein Res., Osaka Univ.)

3: 15 (B-22) Studies on α-lipoic acid-like substances in human urine—Tetsuro Fujita,* Katsumi Mori & Miyoko Nakamura (Dept. of Biochem., Kyoto Prefec. Univ. of Med.)

3: 30 (B-23) Migration of administered C^{14}-labelled riboflavin in rat—Kunio Yagi, Toshiharu Nagatsu & Ikuko Nagatsu-Ishibashi* (Depts. of Biochem. and Anat., Sch. of Med., Nagoya Univ.)

3: 45 (B-24) pH-Fluorescence curves of flavins—Kunio Yagi, Jun Okuda & Rikuko Honda* (Dept. of Biochem., Sch. of Med., Nagoya Univ.)

4: 00 (B-25) Studies on fatty acid esters of flavins. (XV) Dietary survey using rats administered fatty acid esters of FMN—Kunio Kagi, Jun Okuda, Masako Yamada* & Misao Kobayashi (Dept. of Biochem., Sch. of Med., Nagoya Univ.)

4: 15 (B-26) Studies on fatty acid esters of flavins. (XVI) Decomposition of fatty acid esters of FMN by pancreatic lipase—Kunio Yagi, Jun Okuda & Yoshiko Yamamoto (Dept. of Biochem., Sch. of Med., Nagoya Univ.)

4: 30 (B-27) Thiamine-synthesizing system of thiamine-requiring mutants of *E. coli*—Takashi Kawasaki, Akio Iwashima* & Masami Hirabayashi (Dept. of Biochem., Kyoto Prefect. Univ. of Med.)

4: 45 (B-28) Separation of transaminase from rat tissues and its significance—Kōzō Yamada, Shunji Sawaki,* Akira Fukumura & Masaru Hayashi (Dept. of Internal Med., Fac. of Med., Nagoya Univ.)

November 4th (Saturday)

Room C

Morning Session

General Topics

9: 00 (C- 1) Effect of low molecular weight peptides on the pituitary-adrenal system—Genji Shibamoto (Dept. of Biochem., Tokyo Med. Coll.)

9: 15 (C- 2) Identification of the main ethylenediamine condensate of noradrenaline with that of catechol—Toshiharu Nagatsu* & Kunio Yagi (Dept. of Biochem., Sch. of Med., Nagoya Univ.)

9: 30 (C- 3) Effect of hormones on the metabolism of carbohydrate and fatty acid in myocardium—Toshiaki Kishii, Shinsaku Matsumoto,* Yoshio Ito & Tachio Kobayashi (Dept. of Inter. Med., Tokyo Univ. Branch Hosp.)

9 : 45 (C- 4) Photochemical deiodination of thyroxine—Ichiro ISHIKAWA,* Mitsuo SUZUKI & Kiyoshi YAMAMOTO (Inst. of Endocri., Gunma Univ.)

10 : 00 (C- 5) Interrelation between iodination activity and ascorbic acid content in thyroid gland—Mitsuo SUZUKI (Dept. of Physiol., Inst. of Endocri., Gunma Univ.)

10 : 15 (C- 6) Regulation of iodination of thyroglobulin by thyrotropic hormone—Yoichi KONDO* & Nobuo UI (Dept. of Phys. Chem., Inst. of Endocri., Gunma Univ.)

10 : 30 (C- 7) Studies on the thyroid of tadpole : Measurement of absorbance by microscopic spectrophotometer—Sakuji MIYASHITA (Dept. of Physiol. Chem., Inst. of Endocri., Univ. of Gunma)

10 : 45 (C- 8) Studies on the oxidative deamination of 3 : 5 : 3' triiodothyronine by the extracts from mitochondria of rat kidney—Minoru NAKANO (Dept. of Biochem., Sch. of Med., Gunma Univ., & Univ. of Pittsburgh)

11 : 00 (C- 9) Effect of toxohormone on the iron-protoporphyrin chelating enzyme from liver—Motoo HOZUMI* & Mochihiko OHASHI (Cancer Inst., Japan. Found. for Cancer Res.)

11 : 15 (C-10) Some properties of mitochondria from HeLa strain cells grown *in vitro*—Shigeyasu KOBAYASHI,* Bunji HAGIHARA, Masaaki MASUZUMI & Kazuo OKUNUKI (Kobe Hygienic Lab., & Dept. of Bio., Fac. of Sci., Osaka Univ.)

11 : 30 (C-11) Metabolism of glucose and fatty acid in rhodamin sarcoma—Yoshio DOBASHI & Norimasa HOSOYA (Dept. of Biochem., Div. of Health Care, Fac. of Med., Univ. of Tokyo)

11 : 45 (C-12) Effects of high, unsaturated fatty acids on the glucose metabolism of cancer cells—Kozo INABA,* Michio YAMAMOTO & Kozo UTSUMI (Cancer Inst. of Okayama Univ.)

12 : 00 (C-13) Formation of free ethanolamine in hepatoma, and regenerating and normal liver of rat—Masayori INOUE,* Ken HIGASHI, Taijiro MATSUSHIMA & Atsushi OIKAWA (Inst. for Cancer Res., Med. Sch., Osaka Univ.)

12 : 15 (C-14) Effect of dietary protein on turnover rate of the protein in various tissues of rat—Keiichiro MURAMATSU,* Takahiko SATO & Kiyoshi ASHIDA (Lab. of Food & Nutr., Fac. of Agric., Univ. of Nagoya)

Afternoon Session

General Topics

1 : 30 (C-15) Determination of Na, K, Ca, Mg and other elements in serum by atomic absorption spectroscopy—Norihiko HASEGAWA,* Kiyoshi NAKANE, Yahito KOTAKE & Kazuo YASUDA (Central Lab. of Clinl. Chem. & Dept. of Public Health, Sch. of Med., Nagoya Univ., & Hitachi Industl. Co.)

1 : 45 (C-16) Effect of the application of stannous fluoride on teeth on their fluorine content—Seidai MURAI, Takashi KONO & Jiro KANNO (Dept. of Biochem., Sch. of Med., Nihon Univ.)

2 : 00 (C-17) Crystallization of an organic compound containing mercury from shellfish, *Hormoya mutabilis*—Makio UCHIDA, Kiyoshi HIRAKAWA* & Takeshi INOUE (Dept. of Biochem., Med. Sch., Kumamoto Univ.)

2 : 15 (C-18) The behavior of organic acid in relation to the tricarboxylic acid cycle in kidney—Hiroshi ABE, Keinosuke NAKAGAWA* & Makoto SATANI (First Dept. of Med., Med. Sch., Osaka Univ.)

2: 30 (C-19) Metabolism of griseofulvin in human body—Junji NAGAI & Akiko KISHI (Central Clinl. Lab., Kyushu Univ.)

2: 45 (C-20) The metabolism of pyridine nucleotide in the crystalline lens—Kiyoshi UEDA & Tsuneo YUGE (Depts. of Biochem., & Opthalm, Sch. of Med., Kyoto Prefect. Univ.)

3: 00 (C-21) Inhibitory factor for convulsive seizure of ep-mouse present in sera of normal animals—Akitane MORI,* Junji KASAHARA & Sadahiko MASUKAWA (Dept. of Nutr., Okayama Prefect. Univ., & Dept. of Surg., Med. Sch., Okayama Univ.)

3: 15 (C-22) Studies on royal Jelly. (II) Antibody-forming activity and its biological action Akira FUJIMAKI, Hajime SANO* & Akitoshi SHIOYA (Res. Lab. & Chugai Pharm. Co.)

3: 30 (C-23) On the metabolism of hepatic fiber protein. (I)—Chisato HIRAYAMA, Kikuo TOMINAGA, Tsumoru FUKUDA & Toyo YOSHIKAWA (Dept. of Inter. Med., Med. Sch., Kyushu Univ.)

3: 45 (C-24) Variations of the crossing diagrams of the sera of patients against J-protein. (II)—Shojiro NAKAMURA & Shigekata MURAKAWA* (Inst. for Med. Chem., Yamaguchi Med. Coll.)

4: 00 (C-25) Corbicula cholagogue factor. (I) Assay of its function—Hiromi YAMADA (Dept. of Chem., Fac. of Sci., Kyoto Univ.)

4: 15 (C-26) Biochemical mechanism of inflammation. (I) A certain protease and its inhibitor in ARTHUS-type hypersensitivity reaction—Keiji UDAKA,* Masashi KONO & Hideo HAYASHI (First Dept. of Patho., Med. Sch., Kumamoto Univ.)

4: 30 (C-27) Purification of histamine-sensitizing factor from *Bordetella pertussis*—Makoto NIWA (Dept. of Bact., Med. Sch., Osaka City Univ.)

4: 45 (C-28) Effect of 4-amino-5-imidazolecarboxamide on the rat liver injured with CCl_4 —Tōru ISHII, Yasuyuki HAYASHI & Akio GENBA (Tokyo First National Hosp., & Juntendō Univ.)

5: 00 (C-29) Detoxication mechanism of *p*-nitrophenol—Sōhachi ANDO, Yasuho NISHII* & Toshimasa NAKAYAMA (Res. Lab., Chugai Pharm. Co.)

November 4th (Saturday)

Room D

Morning Session

General Topics

9: 00 (D- 1) Reactions between sulfhydryl compounds and protoporphyrinogen—Seiyo SANO* & Norîaki NANJO (Dept. of Public Health, Fac. of Med., Kyoto Univ.)

9: 15 (D- 2) On the cyanide oxidase in human saliva—Koji UENO* & Jiro KANNO (Dept. of Biochem., Sch. of Dent., Nihon Univ.)

9: 30 (D- 3) On the nature of glucosamine dehydrogenase—Fumiko SUZUKI* & Yujiro IMANAGA (Dept. of Chem., Nara Women's Univ.)

9: 45 (D- 4) The enzymatic reduction of phenazine di-N-oxide—Tchan-Gi BAK* & Itiro YOSIOKA (Fac. of Pharm., Osaka Univ.)

10 : 00 (D- 5) Change of heat stability of succinic dehydrogenase by addition of substrate—Kunio YAGI, Kitoku OKADA* & Taro HAYAKAWA (Dept. of Biochem., Sch. of Med., Nagoya Univ.)

10 : 15 (D- 6) Dissociation of artificial Michaelis complex of D-amino acid oxidase—Kunio YAGI & Takayuki OZAWA* (Dept. of Biochem., Sch. of Med., Nagoya Univ.)

10 : 30 (D- 7) D-Kynurenine oxidase—Kinji TSUKADA,* Minoru TASHIRO, Shūhei KOBAYASHI & Osamu HAYAISHI (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

10 : 45 (D- 8) Metabolic product from aromatic compounds by the wood-rottingfungus *Trametes sanguinea.* (III) The reduction of veratric acid to veratric aldehyde by the fungus—Kyoji MINAMI, Toshio FUKUZUMI* & Tetsuo HIYAMA (Fac. of Agric., Univ. of Tokyo)

11 : 00 (D- 9) Purification and properties of mushroom phenol oxidase—Takuro KIMURA* & Shoichiro USAMI (Dept. of Bot., Fac. of Sci., Hokkaido Univ.)

11 : 15 (D-10) Kinetic studies on the action of L-lactate dehydrogenase. (I)—Yumiko UENO & Yasuyuki OGURA* (Dept. of Biochem. & Biophys., Univ. of Tokyo)

11 : 30 (D-11) Kinetic studies on the action of L-lactate dehydrogenase. (II)—Yasuyuki OGURA & Takashi NAKAMURA* (Dept. of Biochem. & Biophys., Univ. of Tokyo)

11 : 45 (D-12) On DPNH peroxidase of *Lactobacillus casei*—Shōji MIZUSHIMA* & Kakuo KITAHARA (Inst. of Applied Microbio., Univ. of Tokyo)

12 : 00 (D-13) Indole acetic acid oxidase activity of wheat peroxidases—Kazuo OKUNUKI, Masamitsu SHIN & Naoi NAKAMURA* (Dept. of Bio., Fac. of Sci., Osaka Univ.)

12 : 15 (D-14) On the catalase isolated from supernatant and mitochondria fractions of liver cells—Michiko YAGI* & Hidematsu HIRAI (Dept. of Biochem., Fac. of Med., Univ. of Tokyo)

Afternoon Session

General Topics

1 : 30 (D-15) On the lactic dehydrogenase of the salivary glands—Junko UYAMA* & Kyoko MOTOI (Dept. of Biochem., Toyo Dent. Coll.)

1 : 45 (D-16) The diaphorase activity and the malic dehydrogenase of the murine leprosy bacillus—Tatsuo MORI (Dept. of Lepr., Res. Inst. for Microbial Diseases, Osaka Univ.)

2 : 00 (D-17) L-Malic dehydrogenase complex from *Mycobacterium avium*—Jiro TOBARI & Tokuji KIMURA (Lab. of Biochem., St. Paul's Univ.)

2 : 15 (D-18) Nitrate and oxygen respiration systems of halotolerant *Micrococcus* 203—Katsuji HORI (Dept. of Chem., Fac. of Sci., Nagoya Univ.)

2 : 30 (D-19) Reduction of neotetrazolium by mitochondria of ascites hepatoma : Effect of coenzyme Q and vitamin K_3—Yoshiro ASO (Dept. of Biochem., Sch. of Med., Chiba Univ.)

2 : 45 (D-20) On coenzyme Q in chromatophore of *Rhodospirillum*—Takashi SUGIMURA (Dept. of Chem., Cancer Inst.)

3 : 00 (D-21) Synthesis of ATP on sonic extracts of *Desulfovibrio desulfricans*—Yoshiko IDA (Dept. of Biophys. & Biochem., Fac. of Sci., Tokyo Univ.)

3 : 15 (D-22) Oxidative phosphorylation coupled with nitrate respiration (I) Evidence for phosphorylation by cell-free extract of *Pseudomonas aeruginosa*—Tateo YAMANAKA, Akihito ŌTA & Kazuo OKUNUKI (Dept. of Bio., Fac. of Sci., Osaka Univ.)

3 : 30 (D-23) Affinity of respiratory substrates for mitochondrial electron transport system —Hakuai KOMAI,* Bunji HAGIHARA & Kazuo OKUNUKI (Dept. of Bio., Fac. of Sci., Osaka Univ.)

3 : 45 (D-24) Mechanism of TTC-reduction in rice plant root—Shooichi MATSUNAKA (National Inst. for Agric. Sci.)

4 : 00 (D-25) Some properties of spinach plostacyanin—Sakae KATOH (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

4 : 15 (D-26) A sulphite reductase from *Aspergillus nidulans*—Akio YOSHIMOTO,* Taro NAKAMURA & Ryo SATO (Inst. for Protein Res., Osaka Univ.)

4 : 30 (D-27) Metabolism of dihydrodiols in animal liver—Tokuro SATO & Haruhisa YOSHIKAWA (Inst. of Public Health, & Fac. of Med., Tokyo Univ.)

4 : 45 (D-28) Studies on acid diazo reaction : Aniline hydroxylase and indole hydroxylase—Yukiya SAKAMOTO, Tōru ŌTANI & Katsuko AKAGI (Dept. of Biochem., Inst. for Cancer Res., & Sch. of Med., Osaka Univ.)

5 : 00 (D-29) Studies on the mechanism of the fission of aromatic ring with enzymes of a bacterium adapted to naphthalene—Hideo OCHIAI,* Hiroshi TANIUCHI, Shiro SENOH & Osamu HAYAISHI (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

November 4th (Saturday)

Room E

Morning Session

Symposium : Structures and Biological Activities of Proteins

9 : 00 (T- 1) Kinetics on the enzymatic action of fragments with peptic activity—Kiyochika TOKUYASU & Masaru FUNATSU (Lab. of Biochem., Fac. of Agric., Kyushu Univ.)

9 : 35 (T- 2) Change of conformation of D-amino acid oxidase in its artificial Michaelis complex fraction—Kunio YAGI & Takayuki OZAWA (Dept. of Biochem., Sch. of Med., Nagoya Univ.)

10 : 10 (T- 3) Binding of myosin A to F-actin—Yuji TONOMURA (Res. Inst. for Catalysis, Hokkaido Univ.)

10 : 45 (T- 4) Tertiary structure and activity of Taka-amylase A and bacterial amylase—Toshizō ISEMURA. Toshio TAKAGI & Akira IMANISHI (Inst. for Protein Res., Osaka Univ.)

11 : 20 (T- 5) The effect of the acetylation of sulfhydryl group on the activity of the enzyme—Kihachiro UEHARA, Kyōichi KOBASHI, Junichi MURASE, Takahiro NISHIMUNE & Seiko MANNEN (Lab. of Biochem., Sch. of Pharm., Osaka Univ.)

12 : 05 (Discussion speech) Chemical structure and enzymatic action of bromelain—Takashi MURACHI & Mieko YASUI (Dept. of Biochem., Sch. of Med., Nagoya City Univ.)

Afternoon Session

Symposium : Continued from Morning Session

1 : 30 (T- 6) Changes in the electronic state of cytochrome c on drying—R. LUMRY, A. SOLBAKKEN & J. SULLIVAN (Dept. of Chem., Univ. of Minesota)

2 : 05 (T- 7) Effect of modified cytochrome c on the activity of cytochrome c oxidase—Kazuo Okunuki & Shigeki Takemori (Dept. of Bio., Fac. of Sci., Osaka Univ.)

2 : 40 (T- 8) The relationship between chemical structure and immunological activity of insulin—Masayasu Kitagawa, Kaoru Onoue & Yuichi Yamamura (Dept. of Biochem., Fac. of Med., Kyushu Univ.)

3 : 15 (T- 9) Structure and enzyme activity of ribonuclease T_1—Kenji Takahashi, Sadako Saigusa & Fujio Egami (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

3 : 50 (T-10) Amino acid composition of ribonuclease from *Bacillus subtilis* and structural comparison with bovine pancreatic ribonuclease—Susumu Nishimura & Hitoshi Ozawa (Found. of Cancer Res., & Inst. for Hard Tissue Res., Tokyo Med. & Dent. Univ.)

4 : 25 (T-11) The chemical structure and enzymic activity of carboxypeptidase-A and -B—Tohoru Tobita, Takeshi Iizuka & Toshio Ando (Dept. of Biophys. Biochem., Fac. of Sci., Tokyo Univ.)

November 5th (Sunday)

Room A

Morning Session

General Topics

9 : 00 (A-30) On the amounts of α-cholestanol and β-cholestanol in blood of the livings suffering some diseases—Hidemoto Oyama* & Jiro Kanno (Dept. of Biochem., Sch. of Dent., Nihon Univ.)

9 : 15 (A-31) On the fate of cholesterol in blood vessels—Tokuro Ichida (Dept. of Biochem., Sch. of Med., Hokkaido Univ.)

9 : 30 (A-32) Cholesterol-binding capacity of serum and its relationship with phospholipids—Seiji Kasuga* & Ikuko Ohtani (Divi. of Biochem., Kanto Teishin Hospital)

9 : 45 (A-33) Determination of α-cholestanol in Ehrlich cancer—Teruo Nebiya,* Jiro Kanno & Hidemoto Ōyama (Dept. of Biochem., Sch. of Dent., Nihon Univ.)

10 : 00 (A-34) Stero-bile acids and bile sterols. (XLII) Stero-bile acids and bile sterols in toad bile—Taro Kazuno & Kyuichiro Okuda (Dept. of Biochem., Sch. of Med., Hiroshima Univ.)

10 : 15 (A-35) Stero-bile acids and bile sterols. (XLIII) Bile salts of the carp—Taro Kazuno & Takahiko Hoshita (Dept. of Biochem., Sch. of Med., Hiroshima Univ.)

10 : 30 (A-36) Stero-bile acids and bile sterols. (XLIV) Stero-bile acids and bile sterols in bull frog bile—Taro Kazuno & Takako Masui (Dept. of Biochem., Sch. of Med., Hiroshima Univ.)

10 : 45 (A-37) On the alloxan Δ^4 cholestenone diabetes—Jiro Kanno, Teruo Nebiya, Hidemoto Ōyama, Seidai Murai, Kōji Ueno & Yasutaka Ishizu (Dept. of Biochem., Sch. of Dent., Nihon Univ.)

11 : 00 (A-38) *In vitro* metabolism of cholesterol-4-C^{14} by rat liver homogenate—Toshiaki Usui (Dept. of Biochem., Fac. of Med., Tottori Univ.)

11 : 15 (A-39) Metabolism of 3-keto-Δ^4-cholenic acid in rat—Michio Ogura (Dept. of Biochem., Fac. of Med., Tottori Univ.)

11 : 30 (A-40) Metabolism of 3α-hydroxy-Δ^7-cholenic acid—Michiro Shimao (Dept. of Biochem., Fac. of Med., Tottori Univ.)

11 : 45 (A-41) Effect of bile acid administration on biosynthesis of cholesterol—Tetsuhiko Sugihara (Dept. of Biochem., Fac. of Med., Tottori Univ.)

12 : 00 (A-42) On the biosynthesis of terpenoids from [2-C^{14}] mevalonic acid with the extract from *Arthemisia monogyma*—Ginzaburo Suzue,* Shizue Oda & Shozo Tanaka (Dept. of Chem., Fac. of Sci., Kyoto Univ. & Dept. of Chem., Kyoto Prefec. Univ.)

12 : 15 (A-43) Effect of parotin and milz on the adrenal cholesterol and vitamin C—Masako Motoi* & Jyunko Uyama (Dept. of Biochem., Tokyo Dent. Coll.)

November 5th (Sunday)

Room B

Morning Session

General Topics

9 : 00 (B-30) Binding between the carcinogenic aminoazo dye and liver proteins of rat.

(VII)—Akira HANAKI*, Masao TAKEUCHI & Hiroshi TERAYAMA (National Inst. of Radiol. Sci., & Dept. Biophys. Biochem., Fac. of Sci., Univ. of Tokyo)

9 : 15 (B-31) Binding of carcinogenic azo-dyes with microsomal nucleoprotein particles. (II) —Toshio YAMADA,* Mitsuo MATSUMOTO & Hiroshi TERAYAMA (Dept. of Biophys. Biochem., Fac. of Sci., Univ. of Tokyo)

9 : 30 (B-32) Ultraviolet carcinogenesis to the skin—Ryohei OGURA* & Fumihide SUENAGA (Dept. of Med. Chem., Sch. of Med., Kurume Univ.)

9 : 45 (B-33) Tyramine as a specific inducer of arylsulphatase synthesis in *Aerobacter aerogenes* —Tokuya HARADA (Inst. of Sci. and Ind. Res., Osaka Univ.)

10 : 00 (B-34) Mechanism of action of mikamycin. (III)—Hideo YAMAGUCHI,* Nobuo TANAKA & Hamao UMEZAWA (Inst. of Applied Microbio., Univ. of Tokyo)

10 : 15 (B-35) Incorporation of S^{35}-methionine into the subcellular fractions of the rat skeletal muscle—Masahiro YAMAGUCHI*, Hiroko TANAKA & Tatuhisa YAMASHITA (Dept. of Nutri. & Biochem., Sch. of Phys. Educa., Juntendo Univ.)

10 : 30 (B-36) Protein biosynthesis by cell-free system of guinea pig cerebral cortex—Yasuo TAKAHASHI, Katsuyoshi MASE, Mei SATAKE & Kikuo OGATA (Brain Res. Inst., Sch. of Med., Niigata Univ.)

10 : 45 (B-37) Incorporation of C^{14}-amino acids into albumin by cell-free systems from rat liver—Ryoji HIROKAWA, Takao TAKAHASHI & Kikuo OGATA (Dept. of Biochem., Sch. of Med., Niigata Univ.)

11 : 00 (B-38) Incorporation of C^{14}-amino acids into γ-globulin and antibody fraction by cell-free system from Immunized rabbit spleen—Shozo OMORI,* Takao TAKAHASHI & Kikuo OGATA (Dept. of Biochem., Sch. of Med., Niigata Univ.)

11 : 15 (B-39) Decrease in ribosomal activity by ultrasonic action and the nature of substances simultaneously released—Kikuo OGATA, Iku WATANABE* & Hiroshi SUGANO (Depts. of Biochem., & Biophys., Fac. of Med., & Dept. of Chem., Fac. of Sci., Niigata Univ.)

11 : 30 (B-40) Fractionation of ribosomes and incorporation of C^{14}-amino acids into the fractions—Hiroyoshi NOHARA (Dept. of Biochem., Sch. of Med., Niigata Univ.)

11 : 45 (B-41) Localization of the amino acids transferred from sRNA into ribosomal protein—Mitsuru TAKANAMI* & Kiichi SEKIGUTI (National Inst. of Animal Health)

12 : 00 (B-42) The nature of glycine-activating enzyme involved in the biosynthesis of silk fibroin—Shigeaki TANAKA*, Iwao SUZUKA & Kensuke SHIMURA (Dept. of Agric. Chem., Tohoku Univ.)

12 : 15 (B-43) Studies on protein synthesis in silk glands—Yoshiaki MIURA, Hiroo ITO, Shigeo TANAKA, Kenhiko MOMOSE & Kiyoshi SUNAGA (Dept. of Physiol. Chem., Sch. of Med., Chiba Univ.)

November 5th (Sunday)

Room C

Morning Session

General Topics

9 : 00 (C-30) On the streaming transparency of the suspension of isolate nuclei—Kaichiro KURODA, Masaaki FUJINO, Goro MANABE & Eiichi KINOSHITA (Dept. of Biochem., Sch. of Med., Tokushima Univ.)

9 : 15 (C-31) Studies on enzymes in egg white. (I) The quantitative changes of carbohydrate and phosphoric acid, and the decomposition of protein, at the begining period of hatching eggs—Susumu HASEGAWA,* Setsuko EZAKI, Masako ISHIKAWA, Itsu TAGUCHI & Yukiko SAKAI (Nagoya Municipal Womens J. Coll., & Shukutoku Coll.)

9 : 30 (C-32) The characteristics of phosphorus metabolism in laying hen. (V) Phosphopeptide in serum and egg-yolk—Kenji MAKINO & Hiroshi NOZAKI (Dept. of Livestock, National Inst. for Agric. Sci.)

9 : 45 (C-33) Studies on the incubation of egg. (II) Labile phosphate at the begining of hatching—Isamu YANAGISAWA,* Hisao AMANO & Toshio ASADA (Dept. of Biochem., Fac. of Med., Toho Univ.)

10 : 00 (C-34) Studies on the incubation of egg. (III) Isolation of a aligopeptide containing pentose—Isamu YANAGISAWA* & Hisao AMANO (Dept. of Biochem., Fac. of Med., Toho Univ.)

10 : 15 (C-35) Studies on the incubation of egg. (IV) Isolation of a polypeptide containing pentose—Isamu YANAGISAWA, Kiichiro KOIKE & Toshiyuki HATASHITA (Dept. of Biochem., Fac. of Med., Toho Univ., & Dept. of Pediat., Tokyo Med. Coll.)

10 : 30 (C-36) Acid-soluble nucleotides of dog leucocytes—Shonosuke SAGISAKA, Masako TAKAHASHI, Masao IKEDA & Kinshi YAGI (Dept. of Physiol., Iwate Med. Coll.)

10 : 45 (C-37) Potassium uptake and adenosinetriphosphate level in human erythrocytes—Haruhisa YOSHIKAWA, Akio SHIMAMUNE, Makoto NAKAO, Yoshinari ISHII, Takashi HASHIMOTO & Masamiti TATIBANA (Dept. of Physiol. Chem. & Nutr., Fac. of Med., Univ. of Tokyo)

11 : 00 (C-38) Formation of ATP analogs in human erythrocytes and their activity for cation active transport—Masamiti TATIBANA, Yoshinari ISHII, Takashi HASHIMOTO & Haruhisa YOSHIKAWA (Dept. of Physiol. Chem. & Nutr., Fac. of Med., Univ. of Tokyo)

11 : 15 (C-39) Nucleic acid and its metabolism in erythrocytes—Makoto NAKAO, Kei, NAGANO, Junko NAKAO, Kenji ADACHI & Toshiko JINBO (Dept. of Biochem., Sch. of Med., Yokohama Univ.)

11 : 30 (C-40) Mechanism of erythrocyte destruction *in vivo*—Makoto NAKAO, Kei NAGANO, Takashi WADA & Kiku NAKAO (Dept. of Biochem., Sch. of Med., Yokohama Univ., & Dept. of Med., Sch. of Med., Gunma Univ.)

11 : 45 (C-41) Formation *in vitro* of hemoglobin and myoglobin from iron, protoporphyrin and globin—Harumi ŌYAMA,* Yoshiki SUGITA, Yoshimasa YONEYAMA & Haruhisa YOSHIKAWA (Dept. of Physiol. Chem. & Nutr., Fac. of Med., Univ. of Tokyo)

12 : 00 (C-42) Heme synthesis in liver—Yoshimasa YONEYAMA,* Takako YASUDA, Harumi OYAMA & Haruhisa YOSHIKAWA (Dept. of Physiol. Chem. & Nutr., Fac. of Med. Univ. of Tokyo)

12 : 15 (C-43) The depressing effect of erythropoiesis by plasma from iron over laded quinea pig—Yoshio YOSHINO & Toshiro KUMAKI* (Dept. of Nutr., Nihon Med. Coll.)

November 5th (Sunday)

Room D

Morning Session

General Topics

9 : 00 (D-30) Chromatography of ionexchange cellulose paper—Yukihiro KAWANISHI, Ichiro

KIMURA, Shuzo SUDA* & Yasuo FUJII (Dept. of Serol., Fac. of Med., Univ. of Tokyo, & Ryukakusan Co.)

9: 15 (D-31) On the properties and purification of pyocin—Makoto KAGEYAMA (Dept. of Biophys. & Biochem., Fac. of Sci., Tokyo Univ.)

9: 30 (D-32) Separation of biologically active fragments after proteolytic degradation of uroparotin—Yosoji ITO, Sahichi OKABE & Masaaki YAMAMOTO (Dept. of Physiol. Chem., Fac. of Pharm. Sci., Univ. of Tokyo, & Res. Lab., Teikoku Hormone Mfg. Co.)

9: 45 (D-33) The cleavage of serum γ-globuline by pepsin and cysteine—Hiroshi NAKAMURA*, Michiko NAKAO, Susumu YAMAMOTO & Tadao KATSURA (Inst. for Infec. Diseases, Univ. of Tokyo)

10: 00 (D-34) The isolation of ophidine from whale muscle—Shigeru TSUNOO, Kazuyoshi HORISAKA, Akihiko MUSASHI & Jiro TANABE (Dept. of Pharm., Showa Med. Sch., & Biochem. Lab., Kobe Womens' Coll. of Pharm.)

10: 15 (D-35) Soluble collagen in bone—Shimpei ARAYA, Shigeru SAITO, Shigeko NAKANISHI* & Yasuhiro KAWANISHI (Dept. of Biochem., Tokyo Med. & Dent. Univ., & Dept. of Serol., Tokyo Univ.)

10: 30 (D-36) Studies on the snake venomes. (I) Column chromatography of protease and lecithinase in Habu venomes—Shigeru KIMURA,* Toshio HAYASHI, Yūichi SAGARA & Takashi KAMIKATAHIRA (Dept. of Biochem., Sch. of Med., Kagoshima Univ.)

10: 45 (D-37) Separation and purification of antigenic substance of *Salmonella flagella*—Ichiro HARUNA* & Tetsuo IINO (Inst. for Virus Res., Kyoto Univ., & National Inst. of Gene.)

11: 00 (D-38) Purification of "c" reactive protein—Tomio OGATA, Ichiro HARA, Yasuhiro KAWANISHI, Jin SATO & Shuzo SUDA (Dept. of Serol., Fac. of Med., Univ. of Tokyo; Tuberc. Lab.; Ryukakusan)

11: 15 (D-39) Isolation and properties of low-density lipoproteins from egg yolk—Hiroshi SUGANO & Iku WATANABE (Dept. of Chem., Fac. of Sci., & Lab. of Biophys. & Biochem., Niigata Univ.)

11: 30 (D-40) On the egg-yolk proteins of several animal species. (II) The chemical nature of the high-density lipoproteins—Yuki ITO, Tatsuzo FUJII & Masae OTAKE (Dept. of Biochem., Gifu Med. Sch.)

11: 45 (D-41) The isolation and characterization of *Chlorella* deoxyribonucleoprotein—Yutaka KOBAYASHI,* Koichi IWAI & Toshio ANDO (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

12: 00 (D-42) Studies on ferritin synthesis. (VIII) Some charactors of non-crystallizable ferritin—Shinji NAKAJIMA & Noriko MURAOKA (Dept. of Nutr., Nippon Med. Sch.)

12: 15 (D-43) Isolation of ferritin from rabbit liver—Noriko MURAOKA & Shinji NAKAJIMA (Dept. of Nutr., Nippon Med. Sch.)

November 5th (Sunday)

Room E

Morning Session

Symposium: Biochemistry of Mucopolysaccharides

A. Biochemistry of Sulfated Mucopolysaccharides

9: 00 (M-1) Comparative biochemistry of the mucopolysaccharides of cartilage—Kimiko

Anno & Nobuko Seno (Dept. of Chem., Fac. of Sci., Ochanomizu Univ.)

9: 15 (M-2) Sulfated mucopolysaccharides of *Elasmobranch* cartilage—Tamotsu Furuhashi,* Kazuko Uchida & Kenji Morita (Seikagaku Kenkyusho Co.)

9: 30 (M-3) Specificity and purification of chondroitin sulfokinase—Eiichi Hasegawa (Biochem. Inst., Kyoto Pref. Univ. of Med.)

9: 45–10: 00 Composite Discussion

B. Biochemistry of Non-Sulfated Mucopolysaccharides

10: 00 (M-4) Isolation and structure of a hexosamine-containing oligosaccharide from the hydrazine-decomposed products of blood-group substance—Jensaku Yoshizawa (Dept. of Med. Chem., Fac. of Med., Tohoku Univ.)

10: 15 (M-5) Glycopeptide of tubercle bacilli—Masahiko Kato* & Edgar Lederer (Toneyama Hosp., & Lab. Biochem., Paris Univ.)

10: 30 (M-6) Syntheses of muramic acid and related compounds with reference to stereostructure of lactic acid moiety—Yoshio Matsushima* & James T. Park (Dept. of Chem., Fac. of Sci., Osaka Univ., & Vanderbilt Univ.)

10: 45–11: 00 Composite Discussion

C. Metabolism of Mucopolysaccharides

11: 00 (M-7) Alteration of mucopolysaccharides in DAB liver tumor—Sachio Tanaka* & Mutsuya Takeda (Dept. of Path., Fac. of Med., Kurume Univ.)

11: 15 (M-8) Synthesis of hexosamine by rat liver—Tōyō Yoshikawa* & Chisato Hirayama (3rd Dept. of Inter. Med., Fac. of Med., Kyushū Univ.)

11: 30–11: 40 Composite Discussion

11: 40 (M-C) *Composite Speech*: Active mucopolysaccharides in blood group substances—Noboru Hiyama (Dept. of Biochem., Fac. of Med., Hirosaki Univ.)

12: 10–12: 20 Composite Discussion

12: 20–12: 30 Conclusive Discussion

November 5th (Sunday)

Afternoon *Joint* Session

(J-1) Determination of amino acid sequences in the active centres of certain enzymes—F. Sanger (Univ. of Cambridge)

(J-2) Studies on Taka-amylase A—Shiro Akabori (Osaka Univ.)

November 6th (Monday)

Room A

Morning Session

General Topics

9 : 00 (A-44) On the phospholipides of the rat skeletal muscle—Tatsuhisa YAMASHITA & Yaeko SOMA (Dept. of Biochem., Sch. of Med., Juntendo Univ.)

9 : 15 (A-45) The lipids in the muscle-cell-membrane-fraction—Tetsuro KONO (Dept. of Agric. Chem., Univ. of Tokyo)

9 : 30 (A-46) Studies on the active principle of tuberculin : Isolation of an unknown aldehyde from the acetone-soluble fat of the human *tubercle bacilli* (strain Aoyama B)—Isaku KASUYA*, Shokai SOFUKU, Sadasuke OBATA, Yoshio TAJIMA & Akira HAGITANI (Lab. of Org. Chem., Fac. of Sci., St. Paul's Univ., & Dept. of Org. Chem., Fac. of Sci., Rikkyo Univ.)

9 : 45 (A-47) Acetone-soluble lipides in caseous materials—Atsunobu YODA (Mikage Branch of Kobe Univ.)

10 : 00 (A-48) Studies on pigments of atypical *Mycobacteria*, photochromogen No. 8 and scotochromogen No. 6—Masakichi MOTOMIYA*, Kikuo MUNAKATA & Oko SATAKE (Res. Inst. for Tuber. and Lepr., Tohoku Univ.)

10 : 15 (A-49) Studies on the glycerophosphatides isolated from BCG—Shoshichi NOJIMA*, Keiko KONDO & Denichi MIZUNO (Microbial Chem., Fac. of Pharm. Sci., Univ. of Tokyo)

10 : 30 (A-50) Chromatographic separation of phospholipids with cellulose-powder-column—Kimiyoshi OHNO, Tadashi SHIMOJO*, Akira YOKOYAMA, Sumiko UEDA & Tomoko YAMADA (Dept. of Biochem., Sapporo Med. Coll.)

10 : 45 (A-51) Studies on lipids of chorioallantoic membrane infected by vaccinia virus—Tamio YAMAKAWA, Nobuo UETA, Seiko KAWAMURA & Takashi KITAMURA (Dept. of Chem., Inst. for Res. in Infect. Diseases.)

11 : 00 (A-52) Studies on malignolipin- (IV)—Takekazu KOSAKI*, Tadao IKEDA, Shinya NAGASAWA, Keishi MURAKI, Noriko NAGAYASU & Toshio SAKA (Dept. of Biochem., Sch. of Med., Mie Prefect. Univ.)

11 : 15 (A-53) Determination of threonine in lipids of Ehrlich tumor—Makoto HAYASHI*, Kunuisuke ONO, Yoshitoku NAKAJIMA & Komei MIYAKI (Inst. of Food-Microbiol., Chiba Univ.)

11 : 30 (A-54) Biochemical studies on complex glycolipids—Tamio YAMAKAWA*, Akira MAKITA, Sachiko YOKOYAMA, Shizuo HANDA & Nobuko KISO (Dept. of Chem., Inst. for Infect. Diseases, Univ. of Tokyo)

11 : 45 (A-55) Fractionation of sulfolipides and isolation of a new enzyme of mucolipide fraction—Sen-ichiroh HAKOMORI & Taiko ISHIMODA (Dept. of Biochem., Cancer Inst., Tohoku Coll. of Pharm.)

12 : 00 (A-56) Glycolipides in fresh-water mussels—Taro HORI*, Katashi HASHIMOTO & Kazuko YAMADA (Dept. of Chem., Fac. of Liberal Arts & Educ., Shiga Univ.)

12 : 15 (A-57) Lysine-containing lipid of *Hyriopsis schlegelii* spermatozoa—Osamu ITASAKA*, Taro HORI & Satoru HIGASHI (Dept. of Chem., Fac. of Liberal Arts & Educ., Shiga Univ.)

Afternoon Session

General Topics

1 : 30 (A-58) Glutarate metabolism—Shosaku NUMA*, Yuzuru ISHIMURA, Yasutomi NISHIZUKA & Osamu HAYAISHI (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

1 : 45 (A-59) A new metabolic pathway of catechol—Yasutomi NISHIZUKA*, Arata ICHIYAMA, Shigenobu NAKAMURA & Osamu HAYAISHI (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

2 : 00 (A-60) A study of the purification and properties of the phospholipase A—Kunihiko SAITO & Donald J. Hanahan (Dept. of Biochem., Univ. of Washington)

2 : 15 (A-61) Studies on the brain phospholipids. (I) Labelling of phospholipid phosphorus in guinea pig brain slices—Kohei HAYASHI*, Tadao KANOH, Shojiro SHIMIZU, Mitsuoki KAI & Saburo YAMAZOE (Dept. of Biochem., Sch. of Med., Gunma Univ.)

2 : 30 (A-62) Studies on the brain phospholipids. (II) Labelling of phospholipid phosphorus in rabbit brain cortex during convulsions—Tadao KANOH*, Kohei HAYASHI, Shozaburo SHIMIZU, Mutsuoki KAI & Saburo YAMAZOE (Dept. of Biochem., Sch. of Med., Gunma Univ.)

2 : 45 (A-63) Organic dialysis of proteolipid separated from beef heart muscle—Saburo FUNAHASHI & Makoto MURAKAMI* (Lab. of Biochem., Dept., of Agric. Chem., Univ. of Tokyo)

3 : 00 (A-64) Biologically active lipid fractions from brain tissues—Sohsaku TSUCHIHASHI*, Katsutaka NAGAI & Yukichi KIMURA (Dept. of Biochem. Cell Res., Inst. for Infect. Diseases, Univ. of Tokyo)

3 : 15 (A-65) The lipides of rat liver cell fractions—Hisao TANI* & Yahito KOTAKE (Dept. of Public Health, Nagoya Univ.)

3 : 30 (A-66) Fat metabolism with tritium-labeled fatty acids—Toshio TAKAI, Yoshimizu KUHARA & Shiki YAMAGUCHI (Dept. of Pedia., Med. Sch., Osaka City Univ.)

3 : 45 (A-67) Gaschromatographic analysis of serum short chain fatty acids by Sr^{90} ionization detector—Yoshiyata TAKAHASHI*, Yasutoshi MUTO* & Kei TANAKA (Dept. of Cli., Fac. of Med., Univ. of Tokyo)

4 : 00 (A-68) CCLF reaction. (IV) Complement consumption phenomena in serum colloidal reaction—Ichiro HARA, Kyoko HOTTA* & Masaharu KUROKAWA (Dept. of Chem., Tokyo Med. & Dent. Univ. & Kitasato Inst.)

4 : 15 (A-69) Fatty acid synthesis in diabetes—Norimasa HOSOYA*, Yoshio DOBASHI, Yohko SUGAWA & Yuriko KAWAI (Dept. of Biochem., Div. of Health Care. Fac. of Med., Univ. of Tokyo)

4 : 30 (A-70) Studies on the lipogenesis in animal tissues under pathological condition. (II) On the desaturation of the fatty acid in diabetic liver and in atheromatous aorta—Yoh IMAI (Dept. of Biochem., Sch. of Med., Hokkaido Univ.)

4 : 45 (A-71) Sulfur-containing lipids in hard-tissues. (III)—Shimpei ARAYA, Nobuhiko KATSURA* & Yoshihiro MARUYAMA (Dept. of Biochem., Fac. of Dent., Tokyo Med. & Dent. Coll.)

5 : 00 (A-72) Lipid metabolism of sweet potato with black rot—Hideo IMASEKI*, Seigo TAKEI & Ikuzo URITANI (Lab. of Biochem., Fac. of Agric., Nagoya Univ.)

November 6th (Monday)

Room B

Morning Session

General Topics

9 : 00 (B-44) Separation of single stranded DNA from double stranded DNA by electrophoresis—Kenichi Matsubara & Yasuyuki Takagi (Dept. of Biochem., Med. Sch., Kanazawa Univ.)

9 : 15 (B-45) Fractionation of oligonucleotides and their ability to induce streptolysin S'—Tatsuya Yamagata & Fujio Egami (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

9 : 30 (B-46) Studies of the properties of streptolysin S'—Jiro Koyama & Fujio Egami (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

9 : 45 (B-47) The structure of glucosylated hydroxymethyl cytidine in T_2, T_4 and T_6 phages—Shigeru Kuno*, E. A. Pratt & I. R. Lehman (Dept. of Biochem., Med. Center, Stanford Univ.)

10 : 00 (B-48) Changes in ultraviolet light sensitivity of germinating *Aspergillus niger* spores—Yoichi Maruyama* & Keiko Hayashi (Inst. of Food Microbiol., & Coll. of Arts & Sci., Chiba Univ.)

10 : 15 (B-49) Free nucleotides in *Chlorella* cells—Tatsuichi Iwamura* & Tamotsu Tanazawa (Tokugawa Inst. of Biol. Res.)

10 : 30 (B-50) Occurrence in blood of a new nucleotide, adenylyl-2, 3-diphosphoglyceric acid Takashi Hashimoto*, Yoshinari Ishii, Masamiti Tatibana & Haruhisa Yoshikawa (Dept. of Physiol. Chem. & Nutr., Fac. of Med., Univ. of Tokyo)

10 : 45 (B-51) Ultraviolet photolysis of nucleosides and nucleotides in the presence of sodium borohydride—Hiroshi Ishihara (Chem. Lab., Nagoya City Univ.)

11 : 00 (B-52) The incorporation of C^{14}-uracil into ψ-uridylic acid—Yoshitaka Kuriki & Yukio Sugino (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

11 : 15 (B-53) Enzymatic formation of histamine-containing nucleotides—Hidemasa Yamasaki, Saburo Muraoka* & Mie Sugiyama (Dept. of Pharm., Med. Sch., Okayama Univ.)

11 : 30 (B-54) The method to get thymine-less mutants of *E. coli*, *Salmonella* and *Bacillus subtilis*—Toshihiko Okada*, Hiromi Sonohara & Jun Honma (Dept. of Biochem., Med. Sch., Kanazawa Univ.)

11 : 45 (B-55) Inhibition by nitrogen mustard on the *in vitro* incorporation of C^{14}-orotic acid into nucleic acids—Akiko Moriyama* & Yoshiaki Miura (Dept. of Physiol. Chem., Med. Sch., Chiba Univ.)

12 : 00 (B-56) Accumulation of acid-soluble deoxyribosidic compounds in mitomycin C-treated bacteria—Isamu Matsumoto*, Makiko Kozaka, Mutsuo Sekiguchi & Yasuyuki Takagi (Dept. of Biochem., Med. Sch., Kanazawa Univ.)

12 : 15 (B-57) Studies on the nucleic acid metabolism. (I)—Kijuro Obara, Sigeru Ono* & Masanobu Oikawa (Dept. of Biochem., Iwate Med. Coll.)

Afternoon Session

General Topics

1 : 30 (B-58) Chromatography of nucleohistones—Yoshiki Ohba (Dept. of Biochem., National

Inst. of Health)

1 : 45 (B-59) Specific, chromatographic interaction between fibroin and silkgland RNA—Satoru Akune & Jun-ichiro Mukai (Agric. Chem. Inst., Kyushu Univ.)

2 : 00 (B-60) Fractionation of ribonuclease core by gel filtration—Hisayuki Ishikura (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo, & Dept. of Biochem., National Inst. of Health)

2 : 15 (B-61) Isolation and characterization of high-turnover RNA of calf thymus nuclei—Atuhiro Sibatani (Rockefeller Inst.)

2 : 30 (B-62) Studies on the acid-soluble phosphorus compounds in connective tissue—Tsunao Tetsuka* & Kunio Konno (Dept. of Biochem., Fac. of Med., Univ. of Tokyo)

2 : 45 (B-63) Novel cytidine diphosphate sugar compounds—Satoshi Okuda, Noboru Suzuki* & Sakaru Suzuki (Dept. of General Educ., Nagoya Univ.)

3 : 00 (B-64) Base composition of the fractionated "soluble" ribonucleic acids—Mitsuhiko Tada* & Mariko Tada (Dept. of Biochem., Sch. of Med., Nagoya Univ.)

3 : 15 (B-65) The nucleotide composition of ribonucleic acids of soluble and particle fractions in several species of bacteria—Kin-ichiro Miura (Inst. of Applied Microbiol., Univ. of Tokyo)

3 : 30 (B-66) Interaction between ribonucleic acid and mercuric chloride—Yoshimi Kawade* & Youko Yamamoto (Inst. for Virus Res., Kyoto Univ.)

3 : 45 (B-67) Effect of ultraviolet irradiation on the permeability and protein synthesis of a *Mycobacterium*—Michio Tsukamura (Obuso National Sanatorium)

4 : 00 (B-68) The relationship between muscle creatine phosphate level and creatinuria in surgical patients—Tadashi Sato (Surgl. Dept., Prefectl. Shinjyo Hosp)

4 : 15 (B-69) An unknown substance in basic ampholytes fraction of normal human urine —Tomoyuki Maekawa (Dept. of Biochem., Sch. of Med., Nagasaki Univ.)

4 : 30 (B-70) Studies on the metabolism of calcium and magnesium. (XXVII) Changes of serum electrolytes of animals illtreated with sonde—Fumimasa Yanagisawa, Kimi Ogasawara* & Satoru Watanabe (Tokyo Metro. Lab. for Med. Sci.)

4 : 45 (B-71) Isolation of S-methylcysteine from human urine—Fumio Tominaga (Dept. of Biochem., Sch. of Med., Nagasaki Univ.)

5 : 00 (B-72) Urinary N-methylhistidine of hens—Shigeru Tsunoo, Kazutaka Horisaka*, Kazue Aso & Shigeru Tokue (Dept. of Pharm., Showa Med. Sch., & Res. Centre, Nihon Nosan Kogyo Co.)

5 : 15 (B-73) Methylguanidine contents in urine of various animals and mechanism of its formation—Iwao Ueda, Sei Shimoyama Kaoru Yokoi*, Hajime Hasegawa & Yasushi Kumon (Dept. of Med. Chem., Osaka Med. Coll.)

November 6th (Monday)

Room C

Morning Session

General Topics

9 : 00 (C-44) Anaerobic energy production of ascaris muscle and action mechanism of anthelmintics—Fujio Obo & Takuro Katsume* (Dept. of Biochem., Fac. of Med., Kagoshima Univ.)

9 : 15 (C-45) On the respiratory metabolism of *B. aneurinolyticus* Kimura et Aoyama—Masayuki Katsumata & Eiichi Sakakibara (Dept. of Hygiene, Osaka Univ. of

Liberal Arts & Educ.)

9 : 30 (C-46) Citric acid metabolism in *Proteus vulgaris*—Akiko KASAMAKI*, Shoji SASAKI & Shoichiro USAMI (Dept. of Bota., Fac. of Sci., Hokkaido Univ.)

9 : 45 (C-47) Levels of pyridine nucleotide coenzymes in lactic acid bacteria—Itaru TAKEBE* & Kakuo KITAHARA (Inst. of Applied Microbiol., Univ. of Tokyo)

10 : 00 (C-48) Effect of spermine on fermentation—Tomomi SAKURADA (Dept. of Biochem., Sch. of Med., Keio Univ.)

10 : 15 (C-49) Effect of biotin on the glucose metabolism in *Brevibacterium flavum*, a glutamate producing bacterium—Isamu SHIIO, Shin-ichiro OTSUKA* & Masahiko TAKAHASHI (Central Res. Lab. of Ajinomoto Co.)

10 : 30 (C-50) Effect of biotin on the cellular permeability of *Brevibacterium flavum*, a glutamate-producing bacterium—Isamu SHIIO*, Shin-ichiro OTSUKA & Masahiro TAKAHASHI (Central Res. Lab. of Ajinomoto Co.)

10 : 45 (C-51) Carbon dioxide fixation in a life cycle of the fission yeast, *Schizosaccharomyces pombe*—Nobundo SANDO (Inst. of Food Microbiol., Chiba Univ.)

11 : 00 (C-52) Biochemical differentiation in mold colonies—Tomomichi YANAGITA* & Fusae KOGANE (Inst. of Food Microbiol., Chiba Univ.)

11 : 15 (C-53) Carboxylic acid metabolism in photosythetic bacteria under light-anaerobic conditions—Shoichi ABE*, Akira MUTO, Hiroshi YAMADA & Goro KIKUCHI (Dept. of Biochem., Sch. of Med., Tohoku Univ.)

11 : 30 (C-54) Glycine metabolism in *Rhodopseudomonas spheroides*—Shigeru TSUIKI & Goro KIKUCHI (Dept. of Biochem., Sch. of Med., Tohoku Univ.)

11 : 45 (C-55) Occurrence of loosely-bound phycobilins in blue-green algae—Yoshihiko FUJITA* & Akihiko HATTORI (Div. of Biosynth. II, Inst. of Applied Microbiol., Univ. of Tokyo)

12 : 00 (C-56) Photochemical reaction involved in processes of phycobilin formation—Akihiko HATTORI* & Yoshihiko FUJITA (Div. of Biosynth. II, Inst. of Applied Microbiol., Univ. of Tokyo)

12 : 15 (C-57) Some observations on the phosphorus metabolism in growing *Chlorella* cells—Shigetoh MIYACHI (Inst. of Applied Microbiol., Univ. of Tokyo)

Afternoon Session

General Topics

1 : 30 (C-58) Comparison of the β-alanine content in wild and black pupae of *Musca domestica* and *Bombyx mori*—Tokuichiro SEKI (Dept. of Gene., Med. Sch., Osaka Univ.)

1 : 45 (C-59) New S-containing amino acids and their derivatives detected during the pydroponic cultivation of garlic—Tomoji SUZUKI, Michiyasu SUGII, Toshio KAKIMOTO* & Nobuo TSUBOI (Fac. of Pharm. Sci., & Inst. for Chem. Res., Univ. of Kyoto)

2 : 00 (C-60) The relationship between tryptophan metabolism and metal chelation—Yukio SHIBATA*, Yuichi MATSUMURA, Tsutomu MIMURA & Hiroshi ITO (Depts. of Food Nutr. & Biochem., Wakayama Med. Coll.)

2 : 15 (C-61) Tryptophan metabolism in the chicken—Yuichi MATSUMURA* & Shujiro ARIYOSHI (Dept. of Biochem., Wakayama Med. Coll.)

2 : 30 (C-62) Isolation of the intermediate of kynurenine metabolism by snake venom—Makio UCHIDA & Kenji UCHIDA (Dept. of Biochem., Med. Sch., Kumamoto Univ.)

2 : 45 (C-63) Studies on the enzymic decomposition and activation of tryptophan—Yoichi MATSUOKA, Norio MIKAMI, Tadao MORIYAMA & Noboru ITOU (Dept. of Biochem., Nara Med. Coll.)

3 : 00 (C-64) Studies on the kynurenine content in the hair of rat—Kazuo HOTTA, Isao ISHIGURO* & Zyunko NAITO (Fac. of Med., Nagoya Univ., & Gifu Coll. of Pharm.)

3 : 15 (C-65) Effect of hypothalamic lesions on some liver enzymes. (II) Tryptophan pyrrolase and transaminase—Takashi SHIMAZU* & Toshihiko SUEMATSU (Inst. for Cancer Res., Osaka Univ.)

3 : 30 (C-66) Tyrosine metabolism of diabetes mellitus—Masashisa WADA, Kanji INAMORI & Hanzo NASU* (1st Dept. of Med., Med. Sch., Osaka Univ.)

3 : 45 (C-67) Studies on the "γ-hydroxyglutamate—pyruvate+glyoxylate" system by C^{14}-labelled compounds in the metabolism of L-hydroxyproline—Kazuoki KURATOMI, Yasuko KOBAYASHI* & Keiko FUKUNAGA (Dept. of Biochem., Sch. of Med., Juntendo Univ.)

4 : 00 (C-68) Enzymatic studies on the catabolism of γ-hydroxy-glutamic acid—Keiko FUKUNAGA*, Shoji KIZUKA & Kazuoki KURATOMI (Dept. of Biochem., Sch. of Med., Juntendo Univ.)

4 : 15 (C-69) Glutamic synthesis in brain tissue—Genkichiro TAKAGAKI* & Keiichi UEMURA (Dept. of Physiol., Keio Univ., & Toho Univ.)

4 : 30 (C-70) Studies on amino acid metabolism in brain tissue *in vivo* using N^{15}-ammonia and C^{14}-glucose—Yasuo TSUKADA*, Shusuke HIRANO, Yutaka NAGATA & Keiichi UEMURA (Dept. of Physiol., Sch. of Med., Toho Univ.)

4 : 45 (C-71) Hydrolysis of γ-hydroxyarginine by arginine desimidase—Satoru MAKISUMI*, Yoshimasa FUJITA & Shinichi SHIBUYA (Dept. of Chem., Fac. of Sci., Kyushu Univ.)

5 : 00 (C-72) Protein metabolism of rat fed on amino acid-imbalanced diet—Akira YOSHIDA & Kiyoshi ASHIDA (Dept. of Agric. Chem., Nagoya Univ.)

November 6th (Monday)

Room D

Morning Session

General Topics

9 : 00 (D-44) The fate of branched chain amino acids in cat—Toshihiko UBUKA, Takahiro KUWAKI & Koji FUKUTOME (Dept. of Biochem., Med. Sch., Okayama Univ.)

9 : 15 (D-45) Crystallization of xanthurenic acid 8-methylether in human urine—Makoto YAMAMOTO*, Tsutomu YATSUHASHI, Akira KOSAKA & Yahito KOTAKE (Dept. of Public Health, Sch. of Med., Nagoya Univ.)

9 : 30 (D-46) Studies on isovalthine—Shinji OOMORI (Dept. of Biochem., Med. Sch., Okayama Univ.)

9 : 45 (D-47) Determination of isovalthine—Kengo KURAHASHI & Toshihiko UBUTA (Dept. of Biochem., Med. Sch., Okayama Univ.)

10 : 00 (D-48) Conversion of allylglycine to allohydroxyproline and threo-hydroxyornithine—Nobuo IZUMIYA*, Yoshimasa FUJITA & Bernhard WITKOP (Dept. of Chem., Fac. of Sci., Kyushu Univ., & N. I. H.)

10 : 15 (D-49) Trinitrophenylation of amino acids and peptides with picryl ether—Kazuo SATAKE, Masaru TANAKA* & Yasuharu TSUZUKIDA (Dept. of Chem., Fac. of Sci., Tokyo Metropolitan Univ.)

10 : 30 (D-50) On a blue pigment obtained from the reaction of phenylalanine with ninhydrin—Naotomo TOMINAGA (Fac. of Literature & Sci., Kagoshima Univ.)

10 : 45 (D-51) On the hydrolysis velocity of dipeptides—Mutumi MURAMATU (Lab. of Protein Chem., Yamaguchi Med. Sch.)

11 : 00 (D-52) Fluorometric determination of urinary kynurenine—Minoru TSUJI* & Yahito KOTAKE (Dept. of Public Health, Sch. of Med., Nagoya Univ.)

11 : 15 (D-53) Behavior of thyroglobulin in density gradient ultracentrifugation—Nobuo UI & Osamu TARUTANI* (Dept. of Phys. Chem., Inst. of Endocri., Gunma Univ.)

11 : 30 (D-54) Studies on the components bound to protein of various organs and body fluid. (I) Screening test by various color reactions—Kaichiro KURODA & Jun NAKATANI* (Dept. of Biochem., Sch. of Med., Tokushima Univ.)

11 : 45 (D-55) On the determination of serum γ-globulin by acriflavine—Yotaro EMURA (Internal Med., Osaka Welfare Pension Hosp.)

12 : 00 (D-56) Studies of lipoprotein with Biuret reaction—Hideo NAKAGAWA (Dept. of Internal Med., 1st National Hosp. of Tokyo)

12 : 15 (D-57) Immunological cross reaction between lysozymes from egg white of hen and duck—Tsunehisa AMANO, Kei FUJIO, Yoshihiko SAIKI, Sohei ARAYA* & Michiko YOSHIMURA (Dept. of Bact., Med. Sch., Osaka Univ.)

Afternoon Session

General Topics

1 : 30 (D-58) Amino acid composition of urease. (I)—Kosei OKI, Gunji USUI, Kiyomichi HANABUSA* & Kiyoshi SEKITA (Dept. of Biochem., Sch. of Med., Keio Univ.)

1 : 45 (D-59) On the amino acid composition of sperm-whale dentinal acid-soluble collagen —Tsuyoshi SAITO* & Atsushi OSHIKANE (Dept. of Biochem., Sch. of Dent., Nihon Univ.)

2 : 00 (D-60) On the homogeneity of protamine and its digestion by pepsin—Shoshi OTA*, Ryuzo HIROHATA, C. C. Yang, Yo IMAI & Teruo ONO (Lab. of Protein Chem., Yamaguchi Med. Sch.; Dept. of Biochem., Kaohsiung Med. Coll.; Dept. of Biochem., Med. Sch., Hokkaido Univ.)

2 : 15 (D-61) Limited proteolysis of native ovalbumin—Kazuo SATAKE, Shin KURIOKA* & Michiko NISHIHARA (Dept. of Chem., Fac. of Sci., Tokyo Metro. Univ.)

2 : 30 (D-62) Structural analyses of one homogeneous component of clupeine by exopeptidases—Masao AZEGAMI* & Toshio ANDO (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

2 : 45 (D-63) Fractionation of arginine-containing oligopeptides—Chizuko NAKAHARA*, Koichi IWAI & Toshio ANDO (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

3 : 00 (D-64) Effects of EDTA and sulfhydryl reagents on the G-F transformation of actin —Yuji TONOMURA & Junko YOSHIMURA* (Res. Inst. for Catalysis, & Dept. of Biochem., Fac. of Sci., Hokkaido Univ.)

3 : 15 (D-65) Denaturation of protein under pressure; bovine serum γ-globulin—Keizo SUZUKI* & Yoshiaki MIYOZAWA (Dept. of Chem., Ritumeikan Univ.)

3 : 30 (D-66) Studies on the subunits of various hemoglobins—Kazuo SATAKE, Shigeru

Sasagawa, Fukuyo Kitamura & Akio Hosoya (Dept. of Chem., Fac. of Sci., Tokyo Metro. Univ.)

3 : 45 (D-67) Hemoglobins from eel—Taro Okazaki*, Kinzaburo Hamada & Nobuko Sumida (Dept. of Biochem., Nippon Med. Univ.)

4 : 00 (D-68) On the primary structure of horse hemoglobin—Genji Matsuda (Max-Planck Inst. for Biochem.)

4 : 15 (D-69) Abnormal human hemoglobins detected in Ube, Yamaguchi Prefecture—Susumu Shibata (Dept. of Cli. Path., Yamaguchi Med. Coll.)

4 : 30 (D-70) Study on the quantitative starch gel electrophoresis—Kunio Matsui* Kei Yaeno (Res. Inst. for Atom. Ene., Osaka City Univ.)

4 : 45 (D-71) Polarography of salivary protein—Kazuo Sanada* & Yukihiko Mishiro (Dept. of Biochem., Nippon Dent. Coll.)

5 : 00 (D-72) Inactivation of serum and seromucoid—Shojiro Sato, Tokuo Amizuka & Kiyomi Sato (Res. Inst. for Tuberc. & Lepr., Tohoku Univ.)

November 6th (Monday)

Room E

Morning Session

Symposium : *Oxygenases*

A. Ring-Cleavage Enzymes 9 : 00—10 : 05

9 : 00 (S-1) Metapyrocatechase—Yutaka Kojima & Osamu Hayaishi (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

9 : 10 (S-2) A new pyrocatechase—Hachiro Nakagawa, Hideo Inoue & Yoshiro Takeda (Dept. of Physiol. Chem., Med. Sch., & Dept. of Biochem., Dent. Sch., Osaka Univ.)

9 : 20 (S-3) Enzymatic ring-cleavage of D-tryptophan—Minoru Tashiro, Kinshi Tsukada, Shuhei Kobayashi & Osamu Hayaishi (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

9 : 35 (S-4) Isolation and identification of the activator of tryptophanpyrrolase—Setsuro Fujii, Takashi Kawachi & Yuichi Yamamura (Dept. of Biochem., Sch. of Med., Kyushu Univ.)

9 : 45~10 : 05 Composite Discussion

Rest, 5 minutes

B. Hydroxylation of Aliphatic Compounds

10 : 10 (S-5) Steroid hydroxylase—Taro Kazuno & Kuichiro Okuda (Dept. of Biochem., Fac. of Med., Hiroshima Univ.)

10 : 20 (S-6) ω-Oxidation of fatty acids—Kazuhiko Wakabayashi, Hiroshi Murakami & Norio Shimazono (Dept. of Biochem., Fac. of Med., Univ. of Tokyo)

10 : 30 (S-7) Oxidation of aliphatic hydrocarbons—Robert K. Gholson (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

10 : 40 (S-8) L-Lysine oxidase—Akira Ichihara (Dept. of Biochem., Dent. Sch., Osaka Univ.

10 : 50~11 : 10 Composite Discussion

Rest, 5 minutes

C. Oxidation of Heterocyclic Compounds

11 : 15 (S-9) Studies on the metabolism of dipicolinic acid (DPA) by microorganism—Yasuo KOBAYASHI & Kei ARIMA (Dept. of Agric. Chem., Fac. of Agric, Univ. of Tokyo)

11 : 25 (S-10) On the hydroxylase reaction of heterocyclic nitrogen compounds—Ryoji ITO, Yukichi HASHIMOTO & Hajime OTAKA (Dept. of Biochem., Sch. of Med., Nihon Univ.)

11 : 40 (S-11) The enzymatic degradation of hemoglobin—Hiroshi NAKAJIMA, Kenji YAMAOKA & Tsutomu TAKEMURA (1st Dept. of Inter. Med., Fac. of Med., Kyushu Univ.)

11 : 50 (S-12) Decomposition of hemoglobin *in vivo*—Goro KIKUCHI & Koh ROKUGO (Dept. of Med. Chem., Fac. of Med., Tohoku Univ.)

12 : 00~12 : 20 Composite Discussion

Afternoon Session

Symposium, Continued from Morning Session

D. Hydroxylation of Aromatic Compounds 1 : 20—2 : 55

1 : 20 (S-13) Hydroxylase of *p*-hydroxybenzoic acid—Keishi YANO, Fumihiro YOSHINAGA & Kei ARIMA (Dept. of Agric. Chem., Fac. of Agric., Univ. of Tokyo)

1 : 35 (S-14) Studies on benzoic acid oxidase system—Takehiko TANAKA, Yoshiichi SAN-NO, Yohichi TITANI & Masami SUDA (Inst. for Protein Res., Osaka Univ.)

1 : 50 (S-15) Enzymatic studies on the hydroxylation of aromatic carboxylic acids—Akira ICHIHARA, Keiichi HOSOKAWA & Kozaburo ADACHI (Dept. of Biochem., Dent. Sch., & Dept. of Physiol. Chem., Med. Sch., Osaka Univ.)

2 : 05 (S-26) The mechanism of double hydroxylation—Hiroshi TANIUCHI & Osamu HAYASHI (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

2 : 20 (S-17) Hydroxylation of aniline catalyzed by liver microsomes—Yoshio IMAI, Yoshiki TAKESUE & Ryo SATO (Inst. for Protein Res., Osaka Univ., & Fac. of Sci., Univ. of Kyoto)

2 : 30~2 : 55 Composite Discussion

Rest, 5 minutes

E. Utilization of Oxygen in vivo 3 : 00~4 : 00

3 : 00 (S-18) On the anaerobic metabolism of aromatic compound in the presence of nitrate —Tairo OSHIMA & Fujio EGAMI (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

3 : 10 (S-19) On the studies of the affinities for oxygen of oxygenases and oxidases—Fuminori KANETSUNA, Yutaka KOJIMA & Osamu HAYAISHI (Dept. of Med. Chem., Fac. of Med., Kyoto Univ.)

3 : 20 (S-20) Mechanism of oxygenation by peroxidase—Isao YAMASAKI (Dept. of Biochem., Fac. of Sci., Tohoku Univ., & Inst. for Applied Elect., Hokkaido Univ.)

3 : 40~4 : 00 Composite Discussion

Rest, 5 minutes

F. Physiological Functions of Oxygenases 4 : 05~5 : 20

4 : 05 (S-21) TPNH-yielding system and steroid hydroxylases in adrenal cortex—Tokuji KIMURA (Dept. of Biochem., Fac. of Sci., Rikkyo Univ.)

4 : 15 (S-22) Metabolism of 5-cyclohexenyl-1, 5-dimethyl barbituric acid—Masazumi TAKESHITA & Shozo TANAKA (Dept. of Pharm. Chem., Fac. of Med., Kumamoto Univ.)

4 : 25 (S-23) Studies on oxidative N-demethylase—Hiroshi TERAYAMA, Akira HANAKI, Mitsuo MATSUMOTO & Tadashi KURIHARA (Dept. of Biophys. & Biochem., Fac. of Sci., Univ. of Tokyo)

4 : 40 (S-24) Studies on liver tryptophan pyrrolase activity of tumor-bearing animal—Yuichi YAMAMURA, Takashi KAWACHI & Setsuro FUJII (Dept. of Biochem., Fac. of Med., Kyusyu Univ.)

5 : 00～5 : 20 Composite Discussion